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Cyclin dependent kinase 2 (CDK2) is a key mediator for EGF-induced cell transformation mediated through the ELK4/c-Fos signaling pathway

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Abstract

Cyclin dependent kinase 2 (CDK2) is a known regulator in the cell cycle control of the G1/S and S/G2 transitions. However, the role of CDK2 in tumorigenesis is controversial. Evidence from knockout mice as well as colon cancer cell lines indicated that CDK2 is dispensable for cell proliferation. In this study, we found that ectopic CDK2 enhances Ras (G12V)-induced foci formation and knocking down CDK2 expression dramatically decreases EGF-induced cell transformation mediated through the down-regulation of *c-fos* expression. Interestingly, CDK2 directly phosphorylates ELK4 at Thr194 and Ser387 and regulates ELK4 transcriptional activity, which serves as a mechanism to regulate *c-fos* expression. In addition, ELK4 is over-expressed in melanoma and knocking down ELK4 or CDK2 expression significantly attenuated the malignant phenotype of melanoma cells. Taken together, our study reveals a novel function of CDK2 in EGF-induced cell transformation and the associated signal transduction pathways. This indicates that CDK2 is a useful molecular target for chemoprevention and therapy against skin cancer.

Keywords

CDK2; ELK4; c-Fos; cell transformation

Introduction

The *c-fos* proto-oncogene, a component of the AP-1 transcription factor complex, is involved in cellular transformation and tumorigenesis (^{1–4}). *c-Fos* transcriptional expression is inducible and begins within minutes after growth factor (e.g., EGF) stimulation. Three

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

major DNA regulatory elements have been identified in the *c-fos* promoter: the sis inducible element (SIE), the serum response element (SRE) and the cAMP response element (CRE) (5–7). The SRE is a pivotal regulatory sequence in the *c-fos* promoter that controls the majority of the signals affecting the *c-fos* promoter (8). The *c-fos* SRE is recognized by the transcription factor ternary complex that is comprised of the SRF (serum response factor) and TCF (ternary complex factor). The TCF belongs to the ETS transcription factor family (5). In the absence of its association with the SRF, the binding of TCF to the SRE of *c-fos* is weak and unstable, whereas the SRF can bind to the SRE of *c-fos* independently of TCF. However, TCF is required for a full and efficient response of the *c-fos* promoter to activators of the RAS/ERKs signaling pathway (9–11).

Cyclin-dependent kinase 2 (CDK2) is a serine/threonine protein kinase, which has a role in the G1/S transition, the initiation of DNA synthesis and the regulation of the exit from S phase. In the G1/S phase, CDK4 and/or CDK6 in a complex with cyclin D initially phosphorylate the retinoblastoma (Rb) protein (12–15). Following an association with E-type cyclin, the CDK2/cyclin E complex completes the phosphorylation of Rb (16–18), which releases and activates the E2F family transcriptional activity, driving cells to the S phase. Besides the phosphorylation of the Rb protein, the activity of the CDK2/cyclin E complex is required for MCM (mini-chromosome maintenance) proteins essential for the initiation of replication (19–21). In late S phase, A-type cyclins that are associated with CDK2 or CDK1 phosphorylate their substrates, including MCMs, Cdc7 or ribonucleotide reductase R2, to control the cell cycle transition from S to G2. In addition, p21Cip1 and p27Kip1, which belong to the Cip/Kip protein family, can form a complex to block CDK2/cyclin E and CDK2/cyclin A kinase activity. On the other hand, CDK2 directly phosphorylates p27Kip1 and induces p27Kip1 degradation through the proteasome pathway, which facilitates the full kinase activity of CDK2/cyclin E to drive cell cycle progression.

Although CDK2 is reportedly over-expressed in many cancer cell lines (22–24), the role of CDK2 in cancer development is still controversial. Knocking down CDK2 by siRNA or antisense oligonucleotides failed to block proliferation of colon cancer cell lines, whereas inhibition of CDK4 caused G1 arrest (25). Although CDK2 is required for germ cell development, CDK2 knockout mice develop normally (26). Proliferation is only slightly affected in CDK2^{-/-} murine embryonic fibroblasts (MEFs). In addition, over-expression of p27Kip1 and p21Cip1 sufficiently blocked cell cycle progression in CDK2^{-/-} MEFs and genetic deletion of CDK2 in *p27kip1*-null mice could not abrogate the development of pituitary tumors, suggesting that CDK2 is a dispensable molecular target of p27Kip1 and p21/Cip1 in cell cycle regulation and tumorigenesis (27). Therefore, the consensus has been that CDK2 is not a very important molecule for regulating cell proliferation, tumorigenesis or as a therapeutic target. However, in this study, we found that CDK2 is required for EGF-induced cell transformation mediated through the regulation of *c-fos* expression. Furthermore, we demonstrated that CDK2 directly phosphorylates ELK4, a member of the TCF family, which provides a mechanism for the regulation of *c-fos* expression by CDK2 in the EGF signal transduction pathway.

Results

CDK2 is required for EGF-induced anchorage independent cell transformation

Although the role of CDK2 in cell cycle regulation is well-known, whether CDK2 is involved in other signal transduction pathways, particularly in oncogenic stimuli-induced cell transformation, is not known. To test the effect of CDK2 on constitutively active Ras (G12V)-induced cell transformation, we co-transfected CDK2, Ras (G12V), or CDK2 plus Ras (G12V) into NIH3T3 cells. The results showed that over-expression of Ras (G12V) induced very clear foci formation, whereas CDK2 alone was not able to efficiently transform NIH3T3 cells (Figure 1A). However, compared with Ras (G12V) alone, co-expression of CDK2 with Ras (G12V) induced more foci formation of NIH3T3 cells (Figure 1A, *right panel*). This suggested that CDK2 might be downstream of the Ras signaling pathway and might play a role in cell transformation. To further investigate the function of CDK2 in cell transformation, we generated HaCaT cells expressing 2 two independent sequences to stably express knockdown CDK2 (Figure 1B, *left panel*). These cells were used to examine the role of CDK2 in EGF-induced cell transformation. Results indicated that EGF-induced anchorage-independent colony formation was markedly reduced in knockdown CDK2 HaCaT cells and confirmed that CDK2 has a critical role in cell transformation (Figure 1B, *right panels*). To study the function of CDK2 in the EGF signaling pathway, we treated knockdown CDK2 HaCaT cells with EGF and the results showed that knocking down CDK2 expression also inhibited EGF-induced c-Fos expression (Figure 1C), but did not affect the phosphorylation of ERK1/2 (Figure 1C). In addition, we also determined the effect of CDK2 on *c-fos* transcriptional expression. The result indicated that *c-fos* mRNA expression was dramatically decreased in CDK2 knockdown cells (Figure 1D) in the presence of EGF stimulation.

ELK4 and CDK2 are novel binding partners

ELK4 (SAP-1a), a member of the TCF family, is a critical molecule in the regulation of *c-fos* transcriptional expression (5, 7, 28). Here, we found that ELK4 is a novel protein-binding partner with CDK2. CDK2-V5 and ELK4-HisG were co-transfected into HEK293 cells and immunoprecipitated (IP) with anti-V5 or anti-HisG. The IP complex included ELK4-HisG and CDK2-V5 (Figure 2A, B). Furthermore, endogenous CDK2 in SK-MEL28 melanoma cells was detected in the complex immunoprecipitated with an ELK4 antibody, but not in the extract immunoprecipitated with the IgG control antibody (Figure 2C).

Thr194 and Ser387 are major sites in ELK4 phosphorylated by CDK2 *in vitro* and *ex vivo*

Based on the results showing that CDK2 interacted with ELK4, we hypothesized that ELK4 might be a novel substrate for CDK2. To determine whether CDK2 could phosphorylate ELK4, we performed an *in vitro* kinase reaction using active CDK2 or JNK1 and GST-ELK4 as substrate and [γ ³²P] ATP. JNK1 is known to phosphorylate ELK4 (28). Therefore, JNK1 was used as a positive control for this experiment. JNK1 and CDK2 both could phosphorylate ELK4 (Figure 3A, *upper panel*), which indicated that ELK4 might be a novel substrate for CDK2. To determine the site(s) of ELK4 that are phosphorylated by CDK2, we designed 8 peptides based on the score predicted by NetPhos 2.0 (Figure 3A, *lower panel*). We then used the different ELK4 peptides as substrates in *in vitro* kinase assays for CDK2.

The results indicated that Thr194 and Ser387 on ELK4 were potential sites that could be phosphorylated by CDK2 (Figure 3B, *upper panel*). We replaced these two potential phosphorylation sites of ELK4 with alanine. The ELK4-WT and ELK4-AA (T194A/S387A) mutant proteins were used as substrates for active CDK2 in an *in vitro* kinase assay. Samples were analyzed by SDS-PAGE and autoradiography and the results showed that, compared with ELK4-WT, the phosphorylation of the double mutant ELK4 was dramatically decreased (Figure 3B, *lower panel*).

Thr194 and Ser387 of ELK4 are within the classical motif (S/TPXR/K) for substrates of CDK2 (Figure 3C). Thus, we determined whether the P-S/T antibody, which recognizes the S/TPXR/K motif, could detect the phosphorylation of ELK4 by CDK2. The *in vitro* kinase assay results indicated that phosphorylation was detectable in ELK4-WT, whereas phosphorylation of ELK4 mutant was weaker (Figure 3D). To study the CDK2 phosphorylation of ELK4 in cells, we co-transfected ELK4-WT and CDK2 into HEK293 cells and the result showed that phosphorylation of ELK4 was dramatically up-regulated (Figure 3E), especially with EGF treatment. In contrast, phosphorylation was substantially decreased in the ELK4-AA mutant cells (Figure 3F). These data indicated that Thr194 and Ser387 are major sites in ELK4 phosphorylated by CDK2 both *in vitro* and *ex vivo*.

Phosphorylation of ELK4 at Thr194 and Ser387 by CDK2 is required for EGF-induced cell transformation

ELK4 (SAP-1a) regulates *c-fos* transcriptional expression through its own transcriptional activity (7, 28-30). Our previous results showed that CDK2 could affect *c-fos* expression and phosphorylate ELK4 at Thr194 and Ser387, which suggests that CDK2 might regulate *c-fos* transcriptional expression through ELK4. To determine whether CDK2 can regulate *c-fos* promoter activity, the *c-fos* luciferase reporter gene, *renilla* luciferase gene and *CDK2-WT* or *CDK2-DN* (*Thr160A*) were co-transfected into HEK293 cells. The data showed that *c-fos* promoter activity increased dose-dependently in cells expressing *CDK2-WT*, but not in cells expressing *CDK2-DN* (Figure 4A). To investigate whether phosphorylation of ELK4 by CDK2 had any effect on ELK4 transcriptional activity, we co-transfected the *c-fos luciferase reporter* with *CDK2* and *ELK4-WT* or *ELK4-AA* (*T194A/S387A*) mutant into HEK293 cells. The ELK4-WT transcriptional activity was increased in a dose-dependent manner with increasing amounts of CDK2 (Figure 4B). In contrast, the double mutant T194A/S387A displayed a dramatically reduced *c-fos* promoter transactivation activity in the presence of CDK2 (Figure 4B). To study the physiological role of ELK4 phosphorylation, we generated HaCaT cells stably expressing ELK4-WT or ELK4-AA (T194A/S387A) mutant (Figure 4C, *left panel*). Cell growth (Figure 4C, *right panel*) and EGF-induced anchorage-independent colony formation (Figure 4D) were both attenuated in the mutant ELK4-AA (T194A/S387A)-expressing cells. EGF-induced c-Fos expression was also decreased in cells expressing mutant ELK4-AA (T194A/S387A) (Figure 4E). Taken together, these data suggest that phosphorylation of ELK4 on Thr194 and Ser387 has a critical role in cell growth and EGF-induced cell transformation.

ELK4 is over-expressed in melanoma and required for tumor growth

Although ELK4 is reported to regulate *c-fos* gene expression, the role of ELK4 in tumorigenesis has not been addressed. To study the clinical relevance of ELK4 in human cancers, we analyzed the TCGA database and found that the *ELK4* gene is amplified or the *ELK4* mRNA is up-regulated in melanoma (Figure 5A) and 16% of patients had changes in ELK4 (Figure 5A). We also examined ELK4 expression in different melanoma cell lines and the results showed that, compared to HaCaT cells, ELK4 was over-expressed in SK-MEL-5 and SK-MEL-28 melanoma cell lines (Figure 5B). To determine whether over-expression of ELK4 is required for tumor cell growth, ELK4 expression was knocked down by specific shRNA in SK-MEL-5 and SK-MEL-28 cell lines (Figure 5C). Cancer cell growth (Figure 5D) and anchorage independent growth (Figure 5D) were both attenuated in knockdown ELK4 cells. Overall, these data indicated that ELK4 is required for maintaining the melanoma malignant phenotype.

Discussion

Most studies regarding CDK2 have focused on cell cycle regulation and, therefore, the role of CDK2 in other signaling pathways is unclear. Cellular transformation is a key process in tumorigenesis, which involves multiple molecules. In this study, we demonstrated that CDK2 has a critical role in cell transformation. Although ectopic CDK2 alone did not induce foci formation in NIH3T3 cells, CDK2 promoted the ability of Ras (G12V)-induced cell transformation (Figure 1A). Knockdown CDK2 significantly attenuated EGF-induced anchorage-independent colony formation of HaCaT cells (Figure 1B), which indicated that CDK2 is involved in EGF-induced cell transformation. These results (Figure 1A, B) indicate that CDK2 activity might be involved in Ras-induced signaling and are consistent with previous reports showing that suppression of Ras function or the Ras/MEK pathway blocks CDK2 activation (^{31, 32}). Our results also showed that knockdown of CDK2 markedly decreased *c-Fos* protein expression as well as its transcriptional expression (Figure 1C, D), but had no effect on phosphorylation of ERK1/2 in EGF-induced signaling (Figure 1C). These data implied that CDK2 regulates *c-fos* expression independent of ERK1/2 activity.

c-Fos transcriptional expression is tightly controlled in normal cells, whereas its over-expression promotes tumorigenesis (^{33–36}). In general, *c-fos* expression is very low but is transiently induced by extracellular stimuli, including serum, growth factors and stress. Although 3 promoter elements are reported to mediate *c-fos* transcriptional expression, SRE (serum response element) is the major element for regulating *c-fos* expression. The *c-fos* SRE is constitutively bound by the SRF (serum response factor) and TCF (ternary complex factor) (^{5, 7}). Three members of the TCF family have been identified, including ELK1, ELK4 (SAP-1A) and NET (SAP-2), which belong to the ETS transcription factor family. These members contain 3 conserved domains, the A domain, which is responsible for DNA binding, the B domain, which is responsible for interacting with SRF to form ternary complexes, and the C domain, which contains multiple S/T-P motifs that are responsible for transcriptional activation. The phosphorylation of TCFs enhances both its DNA binding ability and transcriptional activity induced by stimuli, including EGF, TPA, and UV.

Our data showed that CDK2 interacts with ELK4 (Figure 2) and ELK4 is a novel substrate of CDK2 (Figure 3A). Using peptide screening, we also identified the potential sites (Thr194/ Ser387) in ELK4 phosphorylated by CDK2 (Figure 3B). Phosphorylation of ELK4 was dramatically reduced in the ELK4 T194A/S387A mutant *in vitro* and *ex vivo* (Figure 3D, E, F). Thr194 and Ser387 are consistent with the CDK2 substrate motif (*S/T-PXR/K*) (Figure 3C, *upper panel*). Thr197 is found in ELK4, but not in ELK1 (Figure 3C, *lower panel*). Even though ELK1, ELK4 and ELK3 all belong to the TCF family, each member has its own unique characteristics. Unlike ELK1 and ELK4, NET (ELK3) is a distinctive repressor that exhibits strong transcriptional repressor activity through the NID (net inhibitory domain) and the CID (C-terminal binding protein interaction domain) (³⁷⁻³⁹). ELK1 and ELK4 are both phosphorylated and activated by ERKs and JNKs, but ELK1 is poorly activated by p38, compared with ELK4 (³⁰). Although ELK1 and ELK4 share a high degree of sequence similarity within the ETS domain (80%), these two proteins show different DNA binding specificity (^{40, 41}). In addition, ELK1, but not ELK4, enhances the growth suppressive function of BRCA1a/1b in breast cancer (⁴²).

We showed that ELK4, a transcription factor, is a novel substrate of CDK2. Others have shown that CDK3 and CDK4 both can directly phosphorylate transcription factors, like ATF1 and c-Jun. For example, CDK3 was shown to directly phosphorylate c-Jun at Ser63 and Ser73 and ATF1 at Ser63 in the presence of EGF stimulation (^{43, 44}), and CDK4 is required for c-Jun phosphorylation in immune cells after IL-6 and IL-1 stimulation (⁴⁵). These data indicate that the CDK family of kinases is not only involved in cell cycle regulation but also participates in regulating transcription factor activation.

We investigated the physiological function of ELK4 phosphorylation and the results showed that *c-fos* promoter activity (Figure 4B) as well as c-Fos protein expression (Figure 4E) were dramatically decreased in mutant ELK4 (Figure 4B). As a result, cell growth and anchorage independent transformation were decreased in mutant ELK4 (T194A/S387A) HaCaT cells (Figure 4C, D), which indicated that Thr194 and Ser387 are required for the biological function of ELK4.

Although ELK4 was reported to have an oncogenic role in prostate cancer (⁴⁶⁻⁴⁸), the role of ELK4 in other cancers is unclear. The *TCGA* database showed gene amplification and mRNA up-regulation of *ELK4* in melanoma patient samples (Figure 5A). Our data showed that ELK4 and CDK2 are highly expressed in melanoma cell lines (Figure 5B). Furthermore, the malignant phenotype of SK-MEL-5 and SK-MEL-28 melanoma cell lines, including viability and anchorage independent growth, were dramatically decreased in knockdown ELK4 cells (Figure 5D, E, F). In addition, knocking down CDK2 expression also substantially reduced the malignant phenotype in SK-MEL-5 and SK-MEL-28 melanoma cell lines (Supplementary Figure 1).

Based on this study, CDK2 has a novel biological function in EGF-induced cell transformation and EGF-induced signaling. Although the mechanism explaining how EGF activates CDK2 activity is unclear, our data clearly showed that CDK2 could directly phosphorylate ELK4 at Thr194 and Ser387, which serves as a mechanism for regulation of *c-fos* transcriptional expression. Our data also demonstrated that CDK2 is required for

maintaining the malignant phenotype in melanoma cells, which implies that CDK2 could be a useful chemotherapy or chemoprevention target in skin cancer.

Materials and Methods

Reagents and antibodies

Chemical reagents, including Tris, NaCl, and SDS for molecular biology and buffer preparation were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture media and other supplements were purchased from Life Technologies, Inc. (Rockville, MD). Antibodies to detect the p-S/TP motif and phosphorylated ERK1/2 were obtained from Cell Signaling Technology, Inc. (Beverly, MA). The antibodies against c-Fos, CDK2, ELK4, β -actin, and HA were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-flag was purchased from Sigma-Aldrich. The HisG and V5 antibodies were purchased from Invitrogen (Carlsbad, CA).

Construction of expression vectors

The *pCDNA3.1-CDK2-V5* and *PCDNA4.0-HisG-ELK4* vectors were purchased from Addgene (Cambridge, MA). *GST-ELK4* was sub-cloned from *pCDNA4.0-ELK4* using *EcoRI*, *XhoI*. The *pCDNA4.0-ELK4-T194,S387AA* and *GST-ELK4-T194,S387AA* vectors were constructed from *pCDNA4.0-ELK4-WT* and *GST-ELK4-WT* using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). ELK4 was sub-cloned into the *pBabe* vector using *XbaI* and *XhoI* from the *pCDNA4.0-ELK4* vector. The *pBabe-ELK4-T194,S387AA* mutant was constructed as described above. Lentivirus plasmids containing *sh-CDK2* (#1:TRCN0000039959; #2:TRCN0000039961) and *sh-ELK4* (#1:TRCN0000013886; #2:TRCN0000013887) were purchased from Thermo Scientific (Huntsville, AL).

Cell culture and transfection

All cells came from American Type Culture Collection (ATCC, Manassas, VA) and were cultured at 37°C in a humidified incubator with 5% CO₂ according to ATCC protocols. Cells were cytogenetically tested and authenticated before being frozen. Each vial of frozen cells was thawed and maintained for about two months (10 passages). They were cultured with Dulbecco's modified Eagle's medium (DMEM; Hyclone, San Diego, CA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) and antibiotics at 37°C in a 5% CO₂ incubator. Human embryonic kidney (HEK293) cells, 293T cells, HaCaT cells and SK-MEL-5 and -28 melanoma cells were grown in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin, and cultured at 37°C in a humidified incubator with 5.0% CO₂. NIH3T3 cells were grown in Eagle's Minimum Essential Medium (MEM) supplemented with 10% FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin, and cultured at 37°C in a humidified incubator with 5% CO₂. The cells were maintained by splitting at 90% confluence and media were changed every 3 days. When cells reached 50–60% confluence, transfection was performed using transfectin (Polyplus-Transfection Inc, New York, NY) following the manufacturer's suggested protocol. The cells were cultured for 36–48 h and then proteins were extracted for further analysis.

Lentiviral and retroviral infection

To construct knockdown CDK2 and ELK4 cells, the lentivirus plasmid containing *CDK2* or *ELK4* was transfected into 293T cells together with *PSPAX2* and *PMD2-G*. Viral supernatant fractions were collected at 48 h after transfection and filtered through a 0.45 μm filter. The viral supernatant fractions were infected into the appropriate cells together with 10 $\mu\text{g}/\text{ml}$ polybrene. At 16 h after infection, the medium was replaced with fresh medium containing the suitable concentration of puromycin. The appropriate experiments were performed with these cells, until the control cells (without infection) completely died (usually 2–3 days) in the puromycin medium. For generation of HaCaT cells stably expressing *ELK4-WT* or *ELK4-AA*, the *pBabe-mock*, *pBabe-ELK4-WT* or *pBabe-ELK4-T194,S387AA* plasmid were transfected into 293T cells together with *pCI-VSVG* and *pCI-GPZ*. Viral supernatant fractions were collected at 48 h after transfection and filtered through a 0.45 μm filter. The *pBabe-mock*, *pBabe-ELK4-WT* and *pBabe-ELK4-T194,S387AA* viral supernatant fractions were infected into HaCaT cells together with 10 $\mu\text{g}/\text{ml}$ polybrene. At 16 h after infection, the medium was replaced with fresh medium containing 1 $\mu\text{g}/\text{ml}$ puromycin and cells were then incubated for 6 days.

Reverse transcription-real time PCR

Total RNA was extracted from HaCaT cells infected with *sh-Mock*, *sh-CDK2#1* or *2* after treatment with EGF at various time points using the Qiagen RNeasy kit (Qiagen) according to the manufacturer's instructions. Total RNA (3 mg) was used as a template for the reverse transcription reaction (SuperScript III First-Strand Synthesis System for reverse transcription-PCR, Invitrogen). The *c-fos* primers used were as follows: Forward: 5-actaccactcaccgcagac-3; Reverse 5-ccaggtccgtgcagaagt-3.

MTS Assay

Stably transfected HaCaT and melanoma cells were seeded (1×10^3 /well/100 μl) into 96-well plates, and viability was assessed using an MTS assay kit (Promega, Madison, WI) according to the manufacturer's instructions.

Immunoblotting and immunoprecipitation

Protein samples from cells were extracted with NP-40 cell lysis buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.5% NP-40, and protease-inhibitor cocktail). For immunoblotting, 30 μg of protein were used with appropriate specific antibodies and an alkaline phosphatase (AP)-conjugated secondary antibody and proteins detected by the STORM machine using the Fluorescence/Chemiluminescence mode (Amersham, Piscataway, NJ). For immunoprecipitation, the extracts were combined with agarose A/G beads (50% slurry) by rocking at 4°C overnight. The beads were washed, mixed with 6X SDS-sample buffer, boiled and then resolved by 10% SDS-PAGE. The proteins were detected using the appropriate specific antibodies and an AP-conjugated secondary antibody.

Anchorage independent cell growth

For EGF-induced cell transformation, cells (8×10^3 /mL/well) were exposed to EGF in 1 mL of 0.3% basal medium Eagle (BME) agar containing 10 or 20% FBS. The cultures were

maintained in a 37°C, 5% CO₂ incubator for the appropriate number of days, and cell colonies were scored using a microscope and the Image J (NIH) computer software program. For cancer cell anchorage independent growth, cells (8.0×10³/well) were seeded into 6 well plates with 0.3% BME agar containing 10% FBS and cultured for the appropriate number of days. Colonies were scored using a microscope and the Image J computer program. Statistical analyses were performed using Prism 5.0 statistics software.

***In vitro* kinase assay**

Purified GST-ELK4-WT and GST-ELK4-AA (T194A, S387A) proteins or ELK4 peptides were used as substrates for an *in vitro* kinase assay with active CDK2 (Upstate Biotechnology, Inc). Reactions were carried out at 30°C for 30 min in a mixture containing 50 μM unlabeled ATP and 10 μCi [γ -³²P] ATP, and then were stopped by adding 6X SDS sample buffer. Samples were boiled and then separated by 10% SDS-PAGE or 20% SDS-PAGE (peptide) and visualized by autoradiography, Western blotting, or Coomassie blue staining.

Luciferase assays

293 cells were transfected with *c-fos-Luc* and *SV-40-Renilla-Luc* (Promega, Madison, WI) with CDK2 or ELK4 in the presence of transfection agent. At 30 h after transfection, cells were disrupted in passive lysis buffer and lysates analyzed for firefly and Renilla luciferase activities using the dual luciferase assay kit (Promega).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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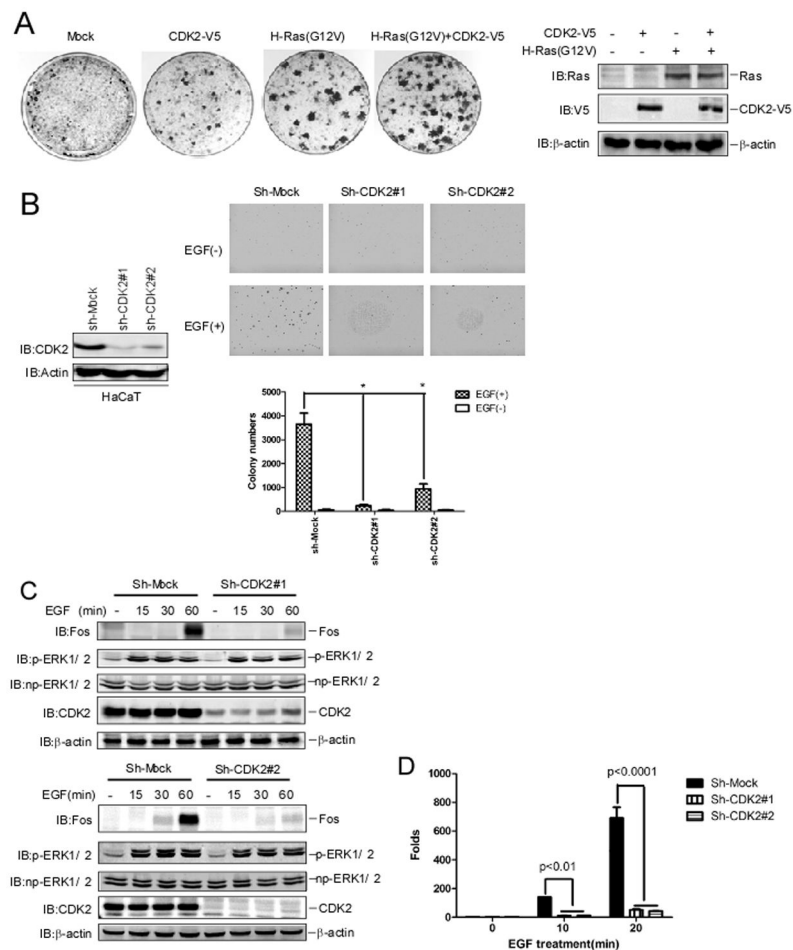


Figure 1. CDK2 plays a critical role in EGF-induced anchorage-independent neoplastic cell transformation mediated through *c-fos*

(A) Ectopic expression of CDK2 enhances Ras (G12V)-induced foci formation. Various combinations of CDK2 and Ras (G12V) were transfected into NIH3T3 cells as indicated and a foci formation assay was conducted following standard protocols as described in *Materials and Methods*. The protein levels of transfected CDK2 and Ras (G12V) were assessed by Western blotting using the indicated antibodies. (B) Knockdown of CDK2 blocks EGF-induced cell transformation. Knockdown of CDK2 in HaCaT cells was performed as described in *Materials and Methods*. Cells were exposed to EGF (20 ng/mL) in 0.3% BME agar containing 20% FBS. The cultures were maintained in a 37°C, 5% CO₂ incubator for 12 d and then colonies were counted using a microscope and the Image J computer software program. Representative photos are shown and data from multiple experiments are expressed as mean values \pm S.D. The asterisk (*) indicates a significant difference between cells expressing *mock* or *sh-CDK* ($p < 0.05$, Student's *t* test). (C) Effect of CDK2 on the EGF signaling pathway. HaCaT cells expressing *sh-CDK2#2* or *sh-CDK2#4* were starved for 36 h and then treated with EGF (20 ng/ml) for various times. Immunoblotting was used to detect phosphorylated and total nonphosphorylated (np) ERK1/2, CDK2 or *c-Fos* protein expression. β -Actin was used to verify equal loading of protein. (D) Effect of CDK2 on *c-fos* transcriptional expression. HaCaT cells stably expressing *mock* or *sh-CDK#1* or *#2* were

starved for 36 h and then treated with EGF (20 ng/ml) for various times as indicated. *c-Fos* transcriptional expression was measured by RT-real time PCR as described in *Materials and Methods*. Data from triplicate experiments are expressed as mean values \pm S.D. Significant differences were evaluated by two-way ANOVA and significant differences are indicated.

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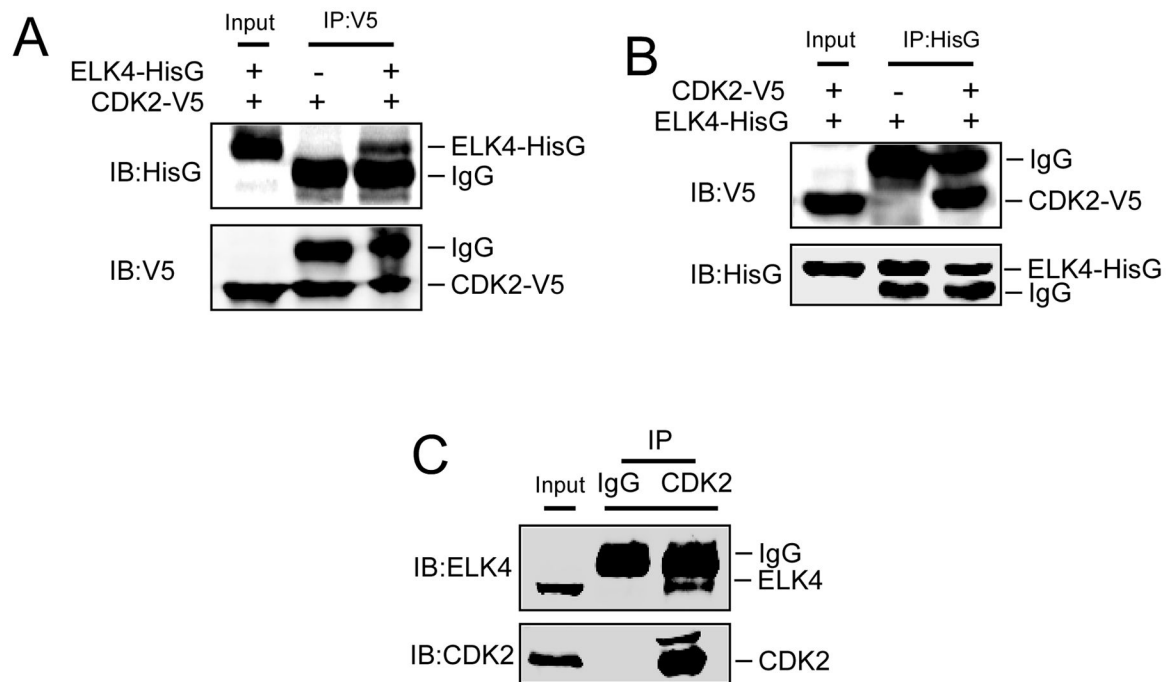


Figure 2. CDK2 binds to ELK4

(A, B) CDK2 interacts with ELK4 *ex vivo*. CDK2-V5 and ELK4-HisG were co-transfected into HEK239 cells. At 36 h after transfection, CDK2-V5 or ELK4-HisG was immunoprecipitated with anti-V5 or anti-HisG. Western blotting was performed using the indicated antibodies. (C) CDK2 interacts with ELK4 *ex vivo*. SK-MEL-28 melanoma cell extracts were used for immunoprecipitation with a CDK2 antibody and control IgG. The immunoprecipitated complex was detected by Western blotting with the indicated antibody. IB: immunoblot; IP: immunoprecipitation.

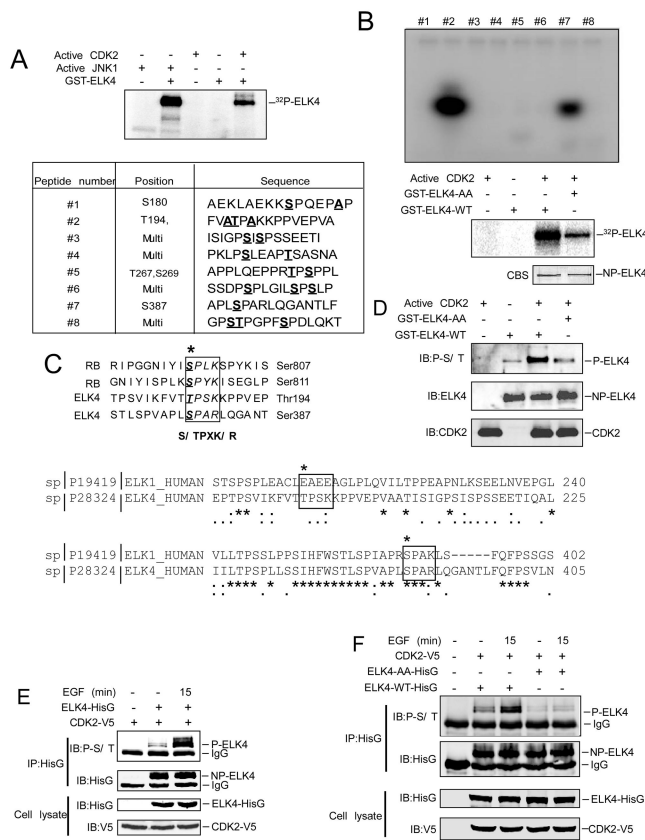


Figure 3. CDK2 phosphorylates ELK4 (Thr194/Ser387)
 (A) CDK2 phosphorylates ELK4 *in vitro*. GST-ELK4 proteins (2.0 μg) were purified from BL21 bacteria and subjected to an *in vitro* kinase assay using active CDK2 (100 ng) or JNK1 (100 ng), 10 μCi of [γ - 32 P] ATP and 50 μM unlabeled ATP, as described in *Materials and Methods*. The reaction mixture was resolved by 10% SDS-PAGE and the phosphorylated ELK4 was visualized by autoradiography (*upper panel*). Amino acid sequences used for peptide synthesis (*lower panel*). (B) Identification of the potential CDK2 phospho-targeted amino acid of ELK4. Peptides (5 μg) synthesized in A were used for an *in vitro* kinase assay with active CDK2 (100 ng) as described in *Materials and Methods*. The reaction mixture was resolved by 20% SDS-PAGE and phosphorylated peptides were visualized by autoradiography (*upper panel*). GST-ELK4-WT and GST-ELK4-AA (T194A/S387A) mutant proteins (2.0 μg) were purified from BL21 bacteria and subjected to an *in vitro* kinase assay using active CDK2 (100 ng) as described in *Materials and Methods*. The reaction mixture was resolved by 10% SDS-PAGE and phosphorylation of ELK4 was visualized by autoradiography (*middle panel*). Coomassie blue staining indicates the respective GST-ELK4-WT and GST-ELK4-AA mutant fusion proteins (*lower panel*). (C) Structure and schematic diagrams of ELK4 amino acid sequences including Thr194 and Ser387. Thr194 and Ser387 are included in the CDK2 substrate motif (S/T-PXR/K) in a manner similar to the RB protein (Ser807/811). The asterisk (*) shows the potential CDK2 phosphorylation sites (*upper panel*). Amino acid alignment between Thr194 of ELK1 and ELK4 is shown (*lower panels*). (D) The phospho-Ser/Thr (p-S/T) antibody recognizes the phosphorylation of ELK4 *in vitro*. An *in vitro* kinase assay was performed using the GST-

ELK4-WT or GST-ELK4-AA mutant protein together with active CDK2 (100 ng) and 200 μ M unlabeled ATP. The phosphorylated ELK4 was detected by the p-S/T antibody and visualized by Western blot. (E, F) Phosphorylation of ELK4 by CDK2 *ex vivo* is partially dependent on EGF treatment. HEK293 cells were transfected with the (E) ELK4-HisG or CDK2-V5 or (F) ELK4-WT-HisG or ELK4-AA-HisG mutant. Cells were cultured for 30 h and treated with EGF (100 ng/ml). The ELK4 proteins were immunoprecipitated with anti-HisG and phosphorylated ELK4 was visualized by Western blot using the p-S/T antibody.

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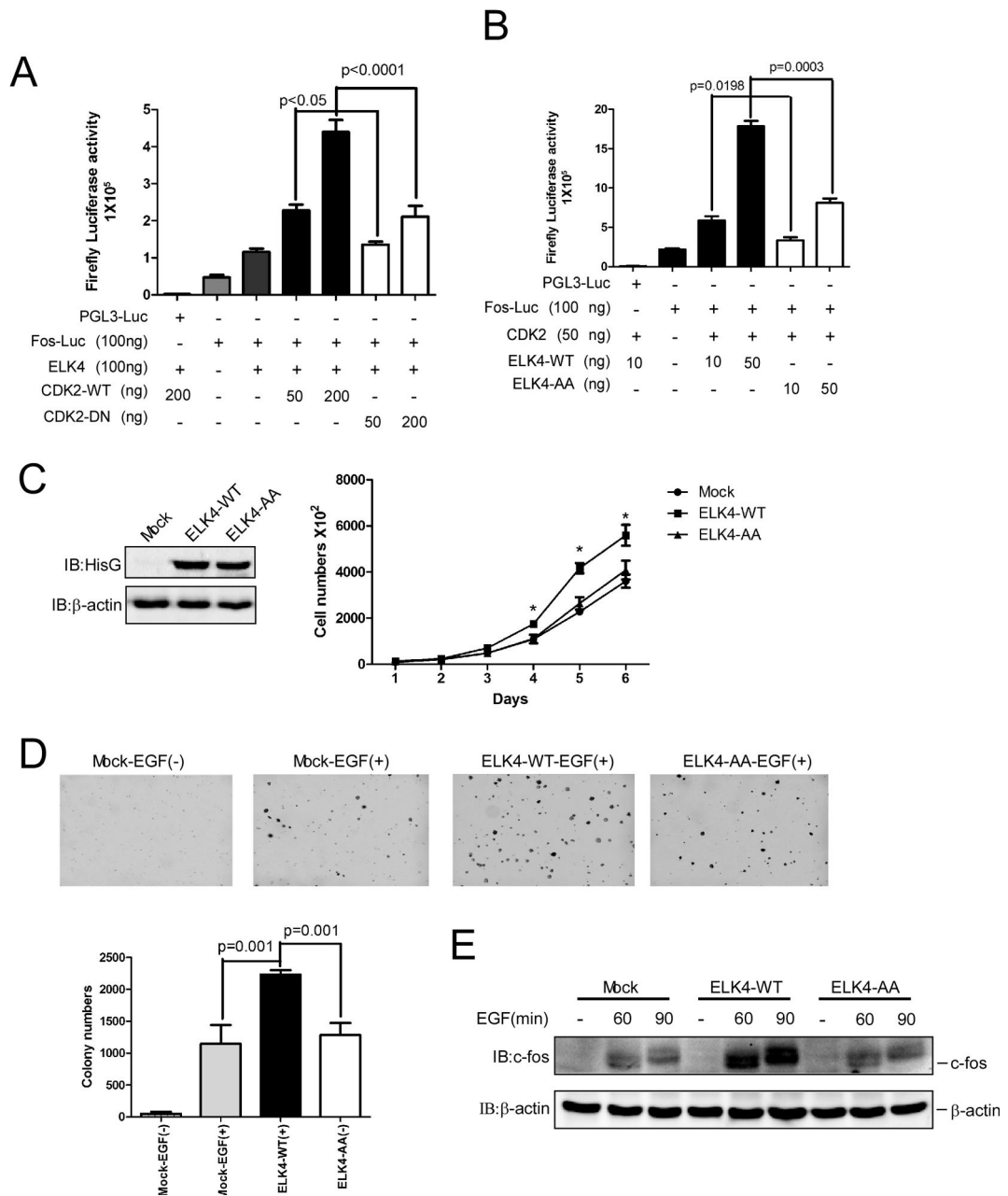


Figure 4. Phosphorylation of ELK4 (Thr194/Ser387) is important for its physiological function (A) CDK2 increases ELK4 transcriptional activity. Various amounts of *CDK2-WT* or *CDK2-DN* (*Thr160Ala*) were transfected with a plasmid mixture containing the *ELK4* or *c-fos-luciferase reporter* gene and the *Renilla luciferase* gene (20 ng) for normalization. At 24 h after transfection, firefly luciferase activity was determined in cell lysates and normalized against *Renilla luciferase* activity. Data from multiple experiments are expressed as mean values \pm S.D. Significant differences were evaluated using a one-way ANOVA and significance level is as indicated. (B) The phosphorylation of ELK4 (Thr194/Ser387) is required for ELK4 transcriptional activation. Various amounts of *ELK4-WT* or *ELK4-AA*

(T194A/S387A) were transfected with a plasmid mixture containing the *CDK2* or *c-fos-luciferase reporter* gene and the *Renila luciferase* gene (20 ng) for normalization. At 24 h after transfection, firefly luciferase activity was determined in cell lysates and normalized against *Renila luciferase* activity. Data from multiple experiments are expressed as mean values \pm S.D. Significant differences were evaluated using a one-way ANOVA and significance level is as indicated. (C) Over-expression of mutant ELK4-T194A/S387A attenuates cell proliferation. HaCaT cells stably over-expressing ELK4-WT or ELK4-T194A/S387A (*left panel*) were constructed as described in *Materials and Methods*. HaCaT cells expressing Mock, ELK4-WT, or ELK4-AA were seeded (1×10^4 per well) into 6-well plates and proliferation was measured. Data from multiple experiments are expressed as mean values \pm S.D. (*right panel*). Significant differences were evaluated using a two-way ANOVA and the asterisk (*) indicates a significant difference ($p < 0.05$). (D) Over-expression of mutant ELK4-T197/S387AA attenuates EGF-induced anchorage independent cell transformation. HaCaT cells expressing Mock, ELK4-WT, or ELK4-AA were exposed to EGF (20 ng/mL) in 0.3% BME agar containing 10% FBS. The cultures were maintained in a 37°C, 5% CO₂ incubator for 12 d and then colonies were counted using a microscope and the Image J computer software program. Data from multiple experiments are expressed as mean values \pm S.D. Significant differences were evaluated using a one-way ANOVA and the respective significant differences are indicated. (E) The mutant ELK4-AA decreases EGF signaling. HaCaT cells expressing Mock, ELK4-WT, or ELK4-AA were starved for 36 h and treated with EGF (20 ng/mL) for various times as indicated. Immunoblotting was performed using specific antibodies.

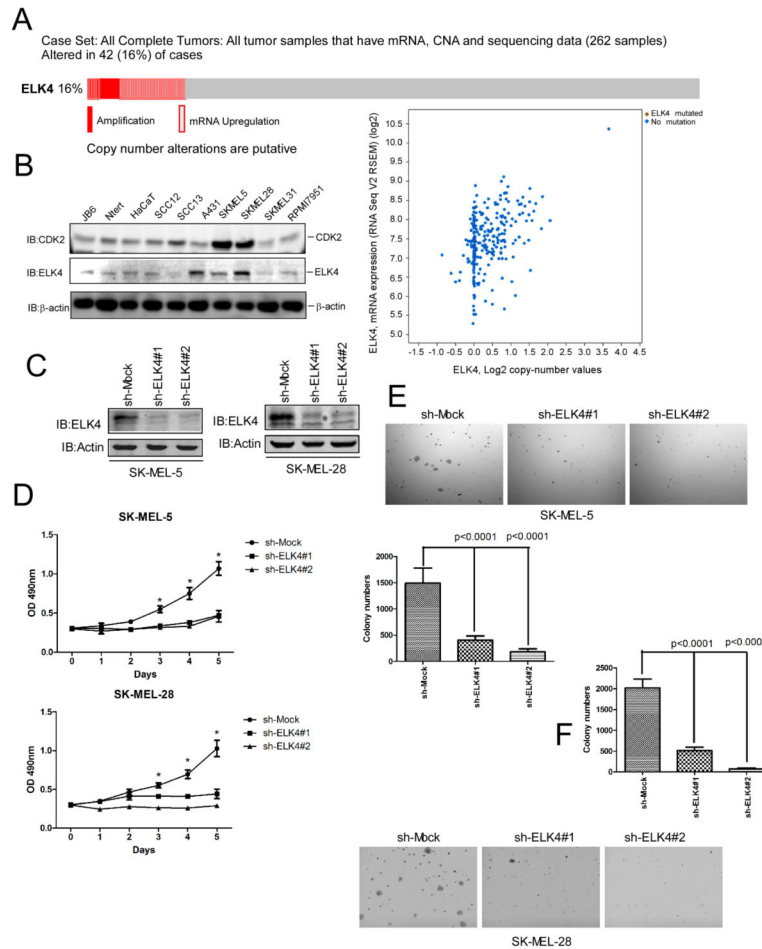


Figure 5. ELK4 is required for melanoma cell growth

(A) Data were obtained from the TCGA database and indicate that *ELK4* is highly over-expressed in cancer. Graphs show the number of *ELK4* amplifications or mRNA (%) (*left upper panel*) and the correlation between *ELK4* gene copy numbers and mRNA expression (*right panel*) in melanomas. (B) ELK4 and CDK2 are over-expressed in various skin cancer cell lines. ELK4 and CDK2 were detected in HaCaT cells and various skin cancer cell lines as indicated. (C) Knockdown of ELK4 attenuates proliferation of SK-MEL-5 and SK-MEL-28 melanoma cells. The cells expressing knockdown ELK4 generated by 2 different sequences were constructed as described in *Materials and Methods*. ELK4 protein expression was assessed by Western blot (WB) as indicated. (D) *Sh-mock*, *sh-ELK4#1* or 2 cells were seeded (1×10^3 per well/100 μ L) into 96-well plates and viability was assessed using the CellTiter96 Aqueous One Solution detection kit. Data from multiple experiments are expressed as mean values \pm S.D. Significant differences were evaluated using a two way ANOVA and the asterisk (*) indicates a significant difference ($p < 0.05$). Knockdown of ELK4 attenuates anchorage independent growth in (E) SK-MEL-5 and (F) SK-MEL-28 melanoma cells. SK-MEL-5 or SK-MEL-28 cells expressing *sh-Mock*, *sh-ELK4#1* or #2 were seeded in 0.3% BME agar containing 10% FBS. The cultures were maintained in a 37°C, 5% CO₂ incubator for 10 d and then colonies were counted using a microscope and the Image J computer software program. Data from multiple experiments are expressed as

mean values \pm S.D. Significant differences were evaluated using a one-way ANOVA and the respective significant differences are as indicated.

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