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ACTL6A suppresses p21^{Cip1} expression to enhance the epidermal squamous cell carcinoma phenotype

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Abstract

Epidermal squamous cell carcinoma (SCC) is a common and highly invasive form of cancer. SCC arises due to ultraviolet light exposure and is associated with increased expression of pro-cancer genes and reduced expression of cancer suppressors. Actin-Like Protein 6A (ACTL6A, BAF53a) is an important protein subunit of the SWI/SNF epigenetic chromatin regulatory complex. ACTL6A is elevated in cancer cells and has been implicated as a driver of cancer cell proliferation and tumor growth. In the present study, we show that ACTL6A drives SCC cell proliferation, spheroid formation, invasion and migration, and that these activities are markedly reduced by ACTL6A knockdown. We further show that ACTL6A expression is associated with reduced levels of the p21^{Cip1} cyclin-dependent kinase inhibitor and tumor suppressor protein. Molecular studies show that ACTL6A interacts with p53 DNA response elements in the p21^{Cip1} gene promoter to suppress p21^{Cip1} promoter activity and mRNA and protein level. Additional studies show that an increase in p21^{Cip1} expression in ACTL6A knockdown cells is required for suppression of the SCC cell phenotype, suggesting that p21^{Cip1} is a mediator of ACTL6A action. We further show that this regulation is p53 independent. These findings suggest that ACTL6A suppresses p21^{Cip1} promoter activity to reduce p21^{Cip1} protein as a mechanism to maintain the aggressive epidermal squamous cell carcinoma phenotype.

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Contributions

Richard L. Eckert and Suruchi Shrestha designed the experiments and wrote the paper

Suruchi Shrestha led the research effort and performed most experiments

Gautam Adhikary and Sivaveera Kandasamy performed additional experiments and critiqued the manuscript

Wen Xu provided technical services and performed experiments.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

ACTL6A; SWI/SNF complex; BAF53A; $p21^{Cip1}$; p53; cancer stem cells; epidermal squamous cell carcinoma

Introduction

Epidermal squamous cell carcinoma (SCC) is among the most common cancers [1, 2]. SCC is treated by surgical excision, but the recurrence and metastatic rates still approach 10% [2]. Because this is a common cancer there are many cases of recurrent therapy-resistant cancer. It is thought that this involves expansion of epidermal cancer stem cells to form rapidly growing, aggressive and invasive tumors [3–5]. Treatment of these cancers has not been successful [6] and so identification of new targets is a major goal.

The SWI/SNF epigenetic regulatory complex controls nucleosome phasing, chromatin remodeling and transcription [7, 8]. The SWI/SNF complex is a large multi-subunit complex that generally acts as a tumor suppressor; however, protein subunits of this complex are frequently mutated or lost in tumors creating conditions that are permissive for cancer development [7]. BAF47, for example, is often lost/mutated in malignant rhabdoid cancer [7] and the Brg1 and Brm proteins, which comprise the catalytic subunits of the SWI/SNF complex, are lost in various cancer cell types [9]. ACTL6A (Actin-Like Protein 6A) is a protein that interacts with the SWI/SNF complex to activate the Brg1 ATPase [10]. However, ACTL6A also acts independent of the SWI/SNF complex to enhance cancer cell survival [11–14]. ACTL6A maintains epidermal stem cell self-renewal to prevent differentiation [15], serves as a c-myc cofactor in cancer stem cells where it acts as an oncogenic driver [16] and is associated with epithelial-mesenchymal transition and metastasis [17–19]. Moreover, ACTL6A overexpression predicts a poor prognosis [19].

ACTL6A has been reported to stabilize the YAP1/TAZ pro-cancer transcriptional regulators [20, 21], and to suppress expression of the $p21^{Cip1}$ tumor suppressor [22–24]. We were interested to understand the role of ACTL6A in epidermal squamous cell carcinoma, as YAP1/TAZ [25, 26] and p53/$p21^{Cip1}$ signaling [27–29] regulate the cancer phenotype. In the present study, we show that ACTL6A enhances the SCC cancer cell phenotype by interacting with and suppressing $p21^{Cip1}$ promoter activity to reduce $p21^{Cip1}$ mRNA and protein. We further confirm that ACTL6A suppression of $p21^{Cip1}$ level is required for optimal cancer cell proliferation, spheroid formation, invasion, migration and tumor formation. Our findings support a model where ACTL6A interacts with the p53 response elements in the $p21^{Cip1}$ promoter to reduce expression via a p53-independent mechanism, and that loss of $p21^{Cip1}$ tumor suppressor enhances the epidermal squamous cell carcinoma phenotype.

Results

ACTL6A maintains $p21^{Cip1}$ level to suppress the cancer cell phenotype

We initiated this study, by examining the impact of suppressing ACTL6A function on cell proliferation, spheroid formation, invasion and migration. Enhanced spheroid formation,

invasion and migration are phenotypic hallmarks of aggressive epidermal cancer cells [3, 25, 26, 30]. Fig. 1A shows the successful knockdown of ACTL6A. Fig. 1B/C/D/E show that ACTL6A loss is associated with reduced SCC-13 cell proliferation, spheroid formation, invasion and migration. To understand how ACTL6A enhances the ECS cell phenotype, we assessed the impact of ACTL6A loss on apoptosis and on $p21^{Cip1}$ cyclin-dependent kinase inhibitor level. Fig. 1F shows that ACTL6A knockdown does not alter procaspase or PARP level, suggesting that ACTL6A does not maintain the pro-cancer phenotype by suppressing apoptosis. In contrast, ACTL6A loss results in a substantial increase in $p21^{Cip1}$ (Fig. 1G), suggesting a possible role for $p21^{Cip1}$. As a complementary approach to study the relationship between ACTL6A and $p21^{Cip1}$, we created ACTL6A knockout cell lines and examined the impact on $p21^{Cip1}$ expression and the cancer phenotype. Fig. 1H confirms ACTL6A loss in each of three clonal ACTL6A knockout cell lines. Fig. 1I/J/K confirms that this is associated with increased $p21^{Cip1}$ expression and shows that stable ACTL6A loss reduces cancer cell proliferation, spheroid formation and invasion.

$p21^{Cip1}$ regulates the ECS cell phenotype

Fig. 1G suggests that ACTL6A may suppress $p21^{Cip1}$ to drive the ECS cell phenotype. To assess the role of $p21^{Cip1}$ as a mediator of ACTL6A action, we performed $p21^{Cip1}$ knockdown experiments. Fig. 2A shows that $p21^{Cip1}$ knockdown does not alter ACTL6A level, suggesting that $p21^{Cip1}$ is a downstream target of ACTL6A. Figs. 2B/C show that loss of $p21^{Cip1}$ increases cell invasion and migration. We next treated ACTL6A knockout cells, which express elevated levels of $p21^{Cip1}$, with $p21^{Cip1}$-siRNA and monitored the impact on invasion and migration. Fig. 2D shows the successful knockdown of $p21^{Cip1}$ and Fig. 2E/F show that reducing $p21^{Cip1}$ level restores cell invasion and migration. These findings confirm that ACTL6A suppresses $p21^{Cip1}$ level as a mechanism to enhance the cancer cell phenotype.

ACTL6A regulates $p21^{Cip1}$ promoter activity

We next examined the mechanism responsible for increased $p21^{Cip1}$ in ACTL6A knockdown cells by studying the impact of ACTL6A on $p21^{Cip1}$ mRNA level and promoter activity. Fig. 2G/H shows an increase in $p21^{Cip1}$ protein and mRNA level following ACTL6A knockdown. To determine if this RNA increase is due to transcriptional regulation, we measured the impact of ACTL6A loss on the activity of p21–2326 Luc, which encodes the full-length $p21^{Cip1}$ gene promoter fused to the luciferase reporter gene. Fig. 2I shows that ACTL6A loss results in increased $p21^{Cip1}$ promoter activity. Fig. 2J shows a map of the $p21^{Cip1}$ promoter, identifying the position of the p53 (p53-1 and p53-2) cis-acting DNA binding elements, that are important mediators of $p21^{Cip1}$ transcription activation and the canonical Sp1 response region (Sp1) which encodes six Sp1 response elements [28, 31]. To determine if the p53 sites are required for ACTL6A regulation of $p21^{Cip1}$ in SCC cells, we measured the impact of ACTL6A loss on activity of wild-type and p53 site-mutant $p21^{Cip1}$ reporter constructs. Loss of ACTL6A results in a substantial increase in luciferase activity for p21–2326 and p21–2326 (p53-Δ2), but not for p21–2326 (p53-Δ1) or p21–2326 (p53-Δ1/Δ2), suggesting that the p53-1 site is required for ACTL6A suppression of $p21^{Cip1}$ promoter activity (Fig. 2K). Consistent with a role for ACTL6A in suppressing $p21^{Cip1}$ promoter

activity, we demonstrate that $p21^{Cip1}$ promoter activity is increased in ACTL6A stable knockout cells (Fig. 2L).

p53 does not mediate ACTL6A action

p53 is known to bind the $p21^{Cip1}$ promoter to activate transcription [29, 31]. To determine if ACTL6A suppresses p53 level to reduce $p21^{Cip1}$, we treated SCC-13 cells with control- or ACTL6A-siRNA and monitored the impact on p53 expression. As shown in Fig. 3A/B, loss of ACTL6A increases p53 mRNA and protein, and this is associated with increased $p21^{Cip1}$ level suggesting that p53 may drive $p21^{Cip1}$ expression in ACTL6A knockdown cells. Moreover, ACTL6A/p53 coprecipitation (Fig. 3C) suggests a cooperative mechanism of action. If p53 drives $p21^{Cip1}$ expression, we would anticipate that interfering with p53 function in ACTL6A knockdown cells would attenuate the increase in $p21^{Cip1}$. The ACTL6A-siRNA/p53-siRNA lane in Fig. 3D shows that suppressing the p53 increase in response to ACTL6A knockdown does not attenuate the increase in $p21^{Cip1}$. Moreover, suppressing p53 does not attenuate the increase in $p21^{Cip1}$ promoter activity, the decrease in cell proliferation or the decrease in invasion and migration (Fig. 3E/F/G/H) observed in ACTL6A knockdown cells. These findings suggest that p53 does not regulate $p21^{Cip1}$ level or the cancer phenotype. To gain additional insight, we measured ACTL6A and p53 interaction at the $p21^{Cip1}$ promoter proximal region (which encodes six Sp1 sites) and at the p53-1 and p53-2 binding sites. We identified ACTL6A binding at the p53-1 and p53-2 sites, and at the proximal promoter, but no p53 interaction on these elements (Fig. 3I). The lack of p53 binding at these sites is consistent with the lack of p53 impact on cancer cell endpoints. This lack of a role for p53 likely relates to the fact that SCC-13 cells express a mutant inactive form of p53 [32].

Role of ACTL6A in HaCaT cells

To assess the role of ACTL6A in a second model system, we used HaCaT cells, an immortalized cell line derived from epidermis [33]. Fig. 4A shows that ACTL6A knockdown increases p53 and $p21^{Cip1}$ in HaCaT cells, and Fig. 4B/C show that ACTL6A loss is associated with reduced spheroid formation (number and size) and migration, suggesting that ACTL6 functions to maintain the HaCaT cell cancer phenotype. We further show (Fig. 4D) that loss of ACTL6A increases $p21^{Cip1}$ promoter activity and that mutation of the p53 binding sites attenuates the increase in promoter activity. To assess the role of p53 in regulating the HaCaT cell phenotype, we compared the impact of ACTL6A and p53 knockdown in HaCaT cells. Fig. 4E shows that ACTL6A knockdown, or simultaneous knockdown of ACTL6A and p53 leads to increased $p21^{Cip1}$ and reduced HaCaT cell migration. In contrast, p53 knockdown did not impact either endpoint. Moreover, ChIP analysis revealed interaction of ACTL6A at the $p21^{Cip1}$ promoter proximal Sp1 region and p53 binding sites. In contrast, p53 binding is not observed (Fig. 4F). These findings suggest that p53 does not modulate ACTL6A regulation of $p21^{Cip1}$ or the cancer phenotype.

Role of ACTL6A in tumor formation

To study the role of ACTL6A in tumor formation, we examined the ability of spheroid-derived wild-type and ACTL6A null cells to form tumors in immune-compromised mice. We show results from two specific ACTL6A knockout cell lines, SCC-13-ACTL6A-

KOc1-10-4 and SCC-13-ACTL6A-KOc1-10-3. Fig. 5A shows that SCC-13-ACTL6A-KOc1-10-4 cells display reduced tumor growth. Fig. 5B shows that loss of ACTL6A is associated with increased p53 and $p21^{Cip1}$, a result that is consistent with the findings from cultured cells. In addition, Fig. 5C show that apoptosis is slightly increased in the ACTL6A null tumors as evidenced by increased presence of cleaved caspase 8 and 9 and cleaved PARP. Similar results were observed in SCC-13-ACTL6A-KOc-1-10-3 cells. Fig. 5D shows a marked reduction in SCC-13-ACTL6A-KOc-1-10-3 cell tumor formation compared to SCC-13 cells, and Fig. 5E/F show that this is associated with increased expression of p53 and $p21^{Cip1}$, and enhanced caspase 3 cleavage.

Discussion

ACTL6A enhances the SCC cell cancer phenotype

ACTL6A has been implicated in regulation of neural progenitor cell proliferation and differentiation [34, 35], maintenance of a stem-like state in epidermal keratinocytes [15] and regulation of EMT, invasion and migration in osteosarcoma and hepatocellular carcinoma [17, 19]. In the present study, we show that transient ACTL6A knockdown reduces SCC cell proliferation, spheroid formation, invasion and migration. These findings were confirmed using multiple clonal ACTL6A knockout cell lines. Biochemical analysis shows that loss of ACTL6A does not promote apoptosis, suggesting that enhanced apoptotic cell death is not a cause of the attenuated cancer phenotype. However, ACTL6A knockdown does result in a marked increase in $p21^{Cip1}$, suggesting that this cyclin-dependent kinase inhibitor/tumor suppressor may be a downstream target that mediates ACTL6A suppression of the cancer cell phenotype. To test this, we monitored the impact of $p21^{Cip1}$ knockdown on cancer cell properties. These studies show that $p21^{Cip1}$ loss results in increased cancer cell invasion and migration. In addition, ACTL6A knockout cell lines express increased levels of $p21^{Cip1}$ and display reduced invasion and migration. Knockdown of $p21^{Cip1}$ in these cell clones resulted in increased cell invasion and migration. Taken together, these findings confirm that the increase in $p21^{Cip1}$ in ACTL6A negative cells attenuates the cancer phenotype, and that $p21^{Cip1}$ is a biologically important downstream mediator of ACTL6A action.

ACTL6A regulates $p21^{Cip1}$ gene transcription

Our studies show that $p21^{Cip1}$ level is increased in ACTL6A knockdown cells, and that the increased $p21^{Cip1}$ level is associated with increased $p21^{Cip1}$ mRNA and $p21^{Cip1}$ gene promoter activity, suggesting transcriptional regulation. ACTL6A loss is also associated with increased p53 levels suggesting that p53, a known positive regulator of $p21^{Cip1}$ expression [36], may be driving $p21^{Cip1}$ gene expression in ACTL6A deficient cells. Further analysis reveals that the p53-1 and/or p53-2 DNA binding sites in the $p21^{Cip1}$ promoter are required for increased promoter activity in ACTL6A knockdown or knockout cells and that mutation of these sites eliminates the increase. These studies strongly suggest a role for p53; however, suppression of the p53 increase observed in ACTL6A knockdown cells does not attenuate the increase in $p21^{Cip1}$. Moreover, modulating p53 level did not attenuate the reduction in cell proliferation, invasion or migration observed in ACTL6A deficient cells. These findings suggest that although ACTL6A interacts with p53 and regulates p53 mRNA and protein level, p53 is not involved as a regulator of $p21^{Cip1}$ expression. Our finding contrasts with a

study showing that ACTL6A reduces $p21^{Cip1}$ level by interacting with p53 to increase p53 acetylation leading to reduced p53 binding to the $p21^{Cip1}$ promoter and reduced promoter activity [22]. Our finding that ACTL6A regulation of $p21^{Cip1}$ expression does not involve p53 is likely because of the presence of mutant p53. In SCC-13 cells p53 glutamate 258 is mutated to lysine (E258K) [32] and in HaCaT cells p53 histidine 179 is mutated to tyrosine (H179Y) and arginine 282 is mutated to tryptophan (R282W) [37]. These p53 DNA binding domain mutations appear to prohibit interaction of these mutants with the p53 response elements. This likely explains why SCC-13 and HaCaT cell-derived p53 do not bind to the $p21^{Cip1}$ promoter p53 response elements, and why manipulating p53 level does not modulate ACTL6A regulation of $p21^{Cip1}$ expression in these cells.

The above findings suggest that ACTL6A acts alone (i.e., without p53), or acts with presently unknown partners, to suppress $p21^{Cip1}$ gene expression. It is possible that other p53 family members, such as ΔNp63α, which are known to regulate $p21^{Cip1}$ expression, may have a role [38]. Identifying co-regulators that act with ACTL6A will require further study.

ACTL6A regulation of tumor formation

To assess the in vivo relevance of these findings, we tested the impact of ACTL6A knockout on tumor formation. An epidermal cancer stem cell (ECS cells) enriched population [3], derived from spheroid cultures of SCC-13 wild-type cells and ACTL6A knockout clones, were injected into immunocompromised mice to monitor the impact of ACTL6A loss on tumor formation. These were selected because they display a highly aggressive phenotype [3]. We observe a dramatic reduction in tumor size for ACTL6A knockout cells. Biochemical analysis of tumor extracts confirms ACTL6A knockout and shows that this is associated with increased levels of p53 and $p21^{Cip1}$. A modest increase in apoptosis is also observed. Thus, in both tumors and cultured cells, ACTL6A loss results in increased $p21^{Cip1}$ levels and this is associated with an attenuated cancer phenotype. The only difference we observe is that ACTL6A loss promotes a modest increase in apoptosis in tumors but not in cultured cells, a finding that could be expected considering the difference in the cell culture versus tumor environment. As apoptosis is only minimally increased in ACTL6A knockout tumors, it is not likely that this is a major cause of reduced tumor growth. The tumor findings are interesting in that the we have previously shown that spheroid-derived cells are enriched for highly aggressive cells that have cancer stem cell properties [3]. The fact that ACTL6A knockout dramatically reduces growth of these tumors argues that ACTL6A has an important role in maintaining the cancer stem cell population. This result is consistent with the reduction in the size of the cancer stem cell-enriched spheroids [3] following ACTL6A knockdown or knockout (Fig. 1K and Fig. 4B).

ACTL6A function in epidermal squamous cell carcinoma

Our findings indicate that ACTL6A loss in epidermal squamous cell carcinoma results in increased transcription of $p21^{Cip1}$ which leads to reduced cancer cell proliferation, spheroid formation, invasion, migration and tumor formation. Moreover, knockdown of $p21^{Cip1}$, in the context of ACTL6A knockout, reduces the response to ACTL6A loss suggesting that $p21^{Cip1}$ is a biologically relevant mediator of ACTL6A action. This suggests that ACTL6A

interacts at the $p21^{Cip1}$ promoter p53 consensus binding sites (p53-1, p53-2) to suppress $p21^{Cip1}$ gene transcription (Fig. 5G). ACTL6A binding to these elements most likely requires other factors, but our studies suggest that p53 is not involved. We note that ACTL6A is also enriched at the proximal promoter region that encodes six Sp1 sites that are known to be involved in regulation of $p21^{Cip1}$ gene expression [31]. This suggests that the proximal promoter may also be involved in ACTL6A regulation of $p21^{Cip1}$. Additional studies will be necessary to assess this possibility. Our findings support the concept that ACTL6A suppresses $p21^{Cip1}$ transcription to reduce $p21^{Cip1}$ mRNA and protein level and that this results in enhanced cancer cell proliferation, spheroid formation, invasion, migration and tumor formation.

Materials and Methods

Reagents

Rabbit anti-ACTL6A (A301–391A) was obtained from Bethyl Laboratories (Montgomery, TX). Rabbit antibodies specific for p53 (9282), $p21^{Cip1}$ (2947), Caspase-9 (NB100–56708), cleaved PARP (556494), caspase-8 (9746) and ChIP grade IgG (2792) were obtained from Cell Signaling Technologies (Danvers, MA). Rabbit anti-caspase-3 (NB100–56708) was obtained from Novus Biologicals (Centennial, CO). Secondary rabbit IgG for immunoprecipitation (NI01) was obtained from Millipore/Sigma (St. Louis, MO). Control (sc-37007), p53 (sc-44218) and $p21^{Cip1}$ (sc-29427) siRNA were obtained from Santa Cruz Biotechnology (Dallas, TX). ACTL6A-siRNA (AM16708) was obtained from Ambion (Philadelphia, PA). The Students t-test was used for statistical analysis. Assays were completed in triplicate and single and double asterisks indicate, respectively, a significant reduction and increase ($p < 0.005$) in response. Five animals per group was selected to give a statistically meaningful outcome based on experience and no animals were excluded from analysis.

Cell culture

SCC-13 are human epidermis derived, tumor forming, squamous cell carcinoma cells [39]. HaCaT cells are immortalized cells derived from human epidermis that do not form tumors [40]. Cells were maintained in growth medium contained DMEM supplemented with 4.5 mg/ml D-glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 U/ml streptomycin and 5 % fetal calf serum. To monitor cell proliferation, 20,000 cells were plated into 35 mm plates in triplicate in growth medium and cell number was monitored from 0 – 3 d. To assay spheroid formation, cells maintained in growth medium were harvested, resuspended in spheroid medium [DMEM/F12 (1:1) containing 2% B27 serum-free supplement, 20 ng/ml EGF, 0.4% bovine serum albumin and 4 mg/ml insulin] and plated at 40,000 cells per 35 mm ultra-low attachment dish [30, 41]. For cell invasion assay, 25,000 cells were plated into Millicell (1 cm diameter, 8 mm pore-size) chambers (353097) atop a 100 μl layer of 300 μg/ml matrigel (354234) [42]. Millicell chambers and matrigel were purchased from BD Biosciences (San Diego, CA). Growth medium containing 1% FCS (top chamber) or 10% FCS (bottom chamber) was added and the ability of cells to pass through the membrane was monitored over 0 – 18 h. The membrane was then fixed with 4% paraformaldehyde and stained with DAPI (D9542, Sigma Aldrich, Milwaukee, WI) and

nuclei of the invading cells were detected by fluorescence microscopy [42]. For migration assay, scratch wounds were created in confluent monolayer cultures of cells using a pipette tip and closure of the wound was monitored from 0 – 24 h.

Electroporation

Cells (1.2 million) were suspended in 100 μl of nucleofection reagent (VPD-1002, Lonza, Williamsport, PA) containing 3 μg of control- or target specific-siRNA and electroporated using the T-018 setting on an AMAXA Electroporator [43]. Pre-warmed growth medium (4 ml) was added and the cells were plated for 48 h in a 60 mm dish. The electroporation was then repeated, and after overnight recovery, the cells were plated to study spheroid formation, invasion and migration [30, 41].

Promoter activity assay

To measure the impact of ACTL6A on $p21^{Cip1}$ promoter activity, cells were double-electroporated with 3 μg control- or ACTL6A-siRNA and plated onto 12 well plates in duplicate. The cells were permitted to attach overnight and then 1 μg reporter plasmid was mixed with 3 μl of Fugene-6 for transfection [31]. The $p21^{Cip1}$ promoter reporter constructs were cloned in pBluescript-Luc (empty vector, EV) as p21–2326, p21–2326 (Δp53-1), p21–2326 (Δp53-2) and p21–2326 (Δp53-1/2). At 24 – 48 h, post-transfection, extracts were prepared for luciferase activity assay. p21–2326, which encodes the intact full length $p21^{Cip1}$ promoter fused to luciferase, was provided by Dr. Bert Vogelstein [44]. The $p21^{Cip1}$ p53 binding site mutant promoter constructs were previously described [31].

Immunological methods

An equivalent amount of protein was electrophoresed on denaturing and reducing 10% polyacrylamide gels and transferred to nitrocellulose. The membrane was blocked by 5% non-fat dry milk and then incubated with appropriate primary and secondary antibody. Antibody binding was visualized using chemiluminescence detection reagents [31]. For immunopreciptation, 200 μg of protein was incubated with 1 μg antibody overnight at 4°C. Washed Pierce Protein A/G agarose (20421, Thermo Scientific, Frederick, MD) was added after 18 h and incubated at 4°C for 4 h. After washing, the beads were boiled in SDS buffer and aliquots were electrophoresed on denaturing and reducing polyacrylamide gels [31].

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described in the Diagenode LowCell# ChIP kit (kch-maglow-G48, Diagenode, Inc., Denville, NJ) protocol. Briefly, one million cells were trypsinized and washed with phosphate buffered saline containing 20 μM sodium butyrate. The cells were crosslinked with 1% formaldehyde at room temperature for 10 min followed by quenching with 125 nM glycine. Crosslinked cells were washed with phosphate buffered saline and lysed with Diagenode lysis buffer supplemented with 1 x protease inhibitor and 20 μM sodium butyrate. The samples were chilled on ice and DNA was sheared using a 550 Sonic Dismembrator (five 30 second pulses at 30% amplitude with 30 seconds between pulses) to obtain 200 – 1000 bp DNA fragments. The samples were diluted in Diagenode buffer containing 20 μM sodium butyrate and 1 x protease inhibitor. ChIP grade antibodies (1 μg)

were incubated with protein G coated magnetic beads for 3 h at 4°C and sheared chromatin (100,000 cells equivalent) was added to the beads and incubated overnight at 4°C. DNA was isolated from the samples the next day using Diagenode DNA isolation buffer with proteinase K. $p21^{Cip1}$ promoter DNA were detected by qPCR using sequence specific primers and LightCycler 480 SYBR green I master mix. ChIP analysis of the $p21^{Cip1}$ promoter included primers that target the proximal Sp1 elements (forward 5'-GCTGGGCAGCCAGGAGCCTG and reverse 5'-CTGCTCACACCTCAGCTGGC), the p53-1 site (forward 5'-GTGGCTCTGATTGGCTTTCTG and reverse 5'-CTGAAAACAGGCAGCCCAAG) and the p53-2 site (forward 5'-CCGAGGTCAGCTGCGTTAGAGG and reverse 5'-AGAACCCAGGCTTGGAGCAGC).

ACTL6A knockout cells

ACTL6A-specific CRISPR guide RNA, forward (5'-caccGGCGATAAAGGCAAACAAGG) and reverse (5'-aaacCCTTGTTTGCCTTTATCGCC), were identified using tools at http://crispr.technology and cloned into the U6-driven pSpCas9(BB)-2A-Puro (PX459) V2.0 vector from Addgene. The vector (3 μg) was electroporated into SCC-13 cells using the AMAXA electroporator and at 48 h pos-electroporation the cells were treated with 2 μg/ml puromycin for 24 h followed by selection of single cell clones by dilution cloning.

qRT-PCR

RNA was isolated using Illustra RNAspin Mini kit (25050070, GE Healthcare, Chicago, IL), reverse-transcribed and quantified using the LightCycler 480 PCR system (Roche Life Science, Branford, CT). Specific PCR primers were used to quantify the transcript level using LightCycler 480 SYBR Green I, and signals were normalized to cyclophilin A. The primers include $p21^{Cip1}$ forward (5'-CGTCTGCAACCACAGGGATTTCTT-3') and reverse (5'-TGTTGATTGTCACATGCTTCCGGG-3'), and p53 forward (5'-TAACAGTTCCTGCATGGGCGGC-3') and reverse (5'-AGGACAGGCACAAACACGCACC-3').

Tumor xenografts

Cells (200,000) were re-suspended in 200 μl of phosphate buffered saline containing 30% matrigel and 100 μl was injected subcutaneously into each front flank of five 8-week-old NOD/*scid*IL2 receptor γ chain knockout mice (NSG) per treatment group. Tumor growth was monitored by measuring tumor diameter and calculating tumor volume = $4/3\pi \times (\text{diameter}/2)^3$. Tumor samples were harvested to prepare extracts for immunoblot [45].

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Abbreviations:

ACTL6A	Actin-Like Protein 6A
SCC	epidermal squamous cell carcinoma

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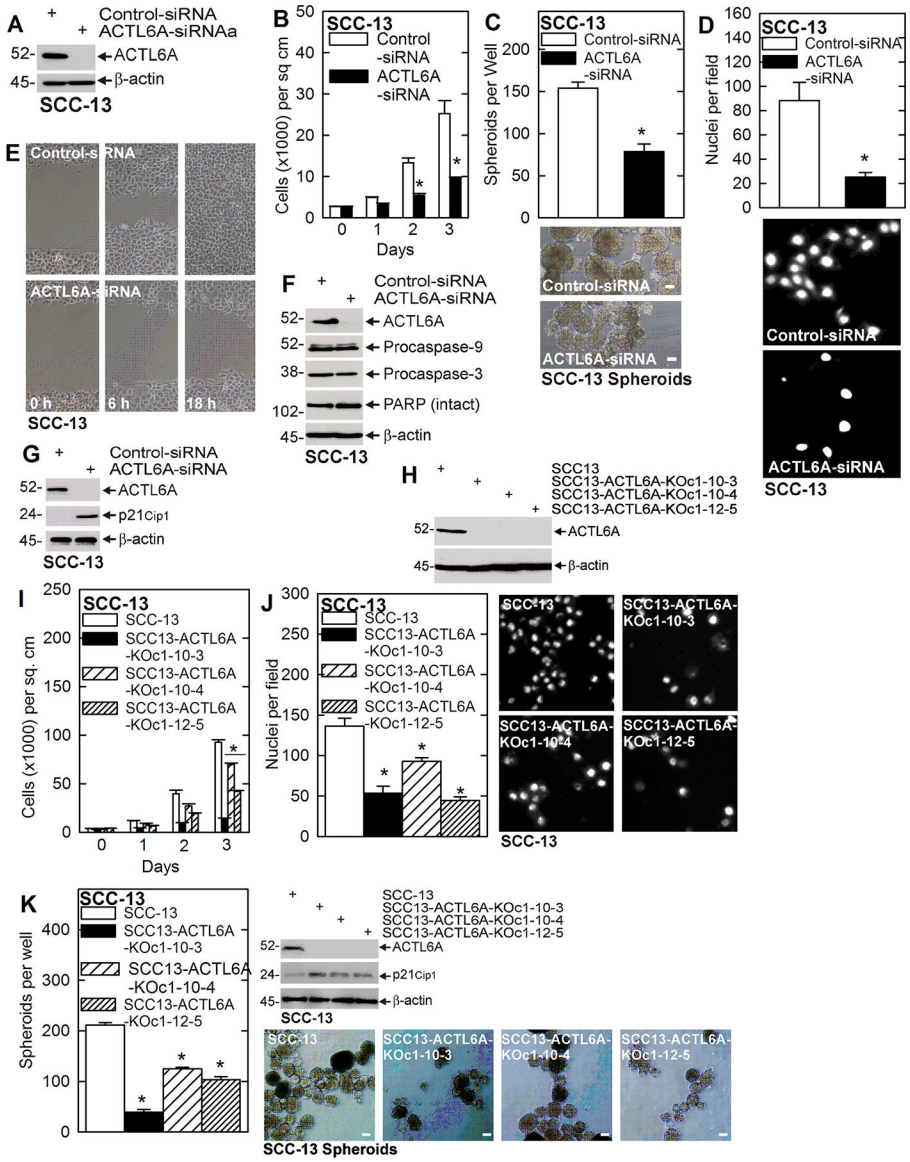

Fig. 1.

ACTL6A knockdown attenuates the cancer cell phenotype **A** Successful siRNA knockdown of ACTL6A. **B/C/D/E** ACTL6A knockdown reduces SCC-13 cell proliferation, spheroid formation, invasion and migration. **F** ACTL6A knockdown does not impact apoptosis marker levels. **G** ACTL6A knockdown results in increased $p21^{Cip1}$ levels. **H/I/J/K** ACTL6A knockout cells display increased $p21^{Cip1}$ level which is associated with reduced cell proliferation, invasion and spheroid formation.

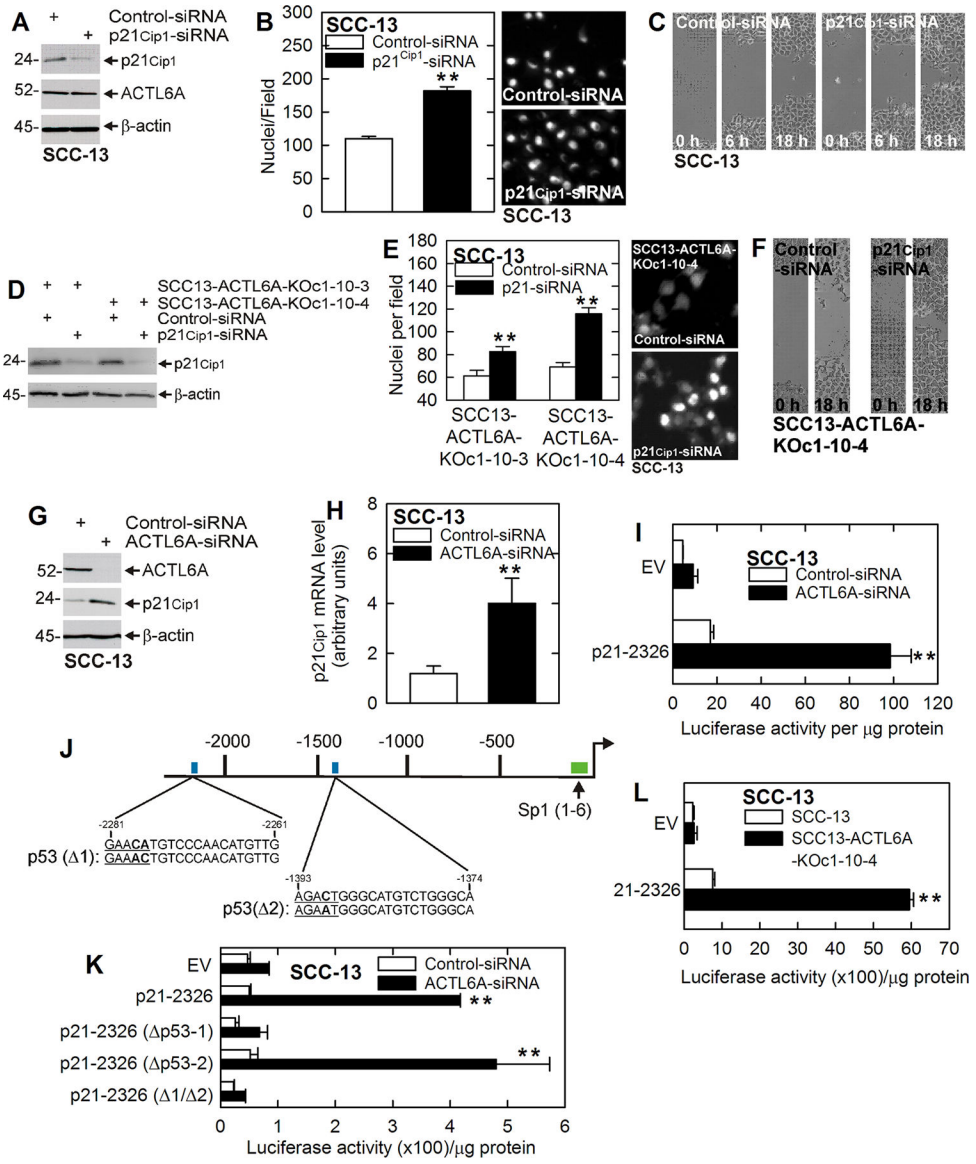

Fig. 2.

p21^{Cip} mediates ACTL6A action **A** Successful siRNA knockdown of p21^{Cip1} with absence of impact on ACTL6A level. **B/C** p21^{Cip1} knockdown enhances invasion and migration. **D** Successful p21^{Cip1} knockdown in ACTL6A knockout cells. **E/F** p21^{Cip1} knockdown in ACTL6A-null cells enhances cell invasion and migration. **G/H** ACTL6A knockdown increases p21^{Cip1} protein and mRNA levels. **I** ACTL6A knockdown increases activity of the full-length p21^{Cip1} promoter. **J** p21^{Cip1} promoter map showing the full-length promoter (nucleotides −2326/−1) including the proximal Sp1 response region, the two p53 response elements and the start of transcription (arrow). The sequence of the wild-type and mutant p53 elements are also shown. **K** The p53-1 site is required for the increase in p21^{Cip1} promoter activity in response to ACTL6A knockdown. **L** p21^{Cip1} promoter activity is elevated in ACTL6A-knockout cells lines as compare to wild-type cells.

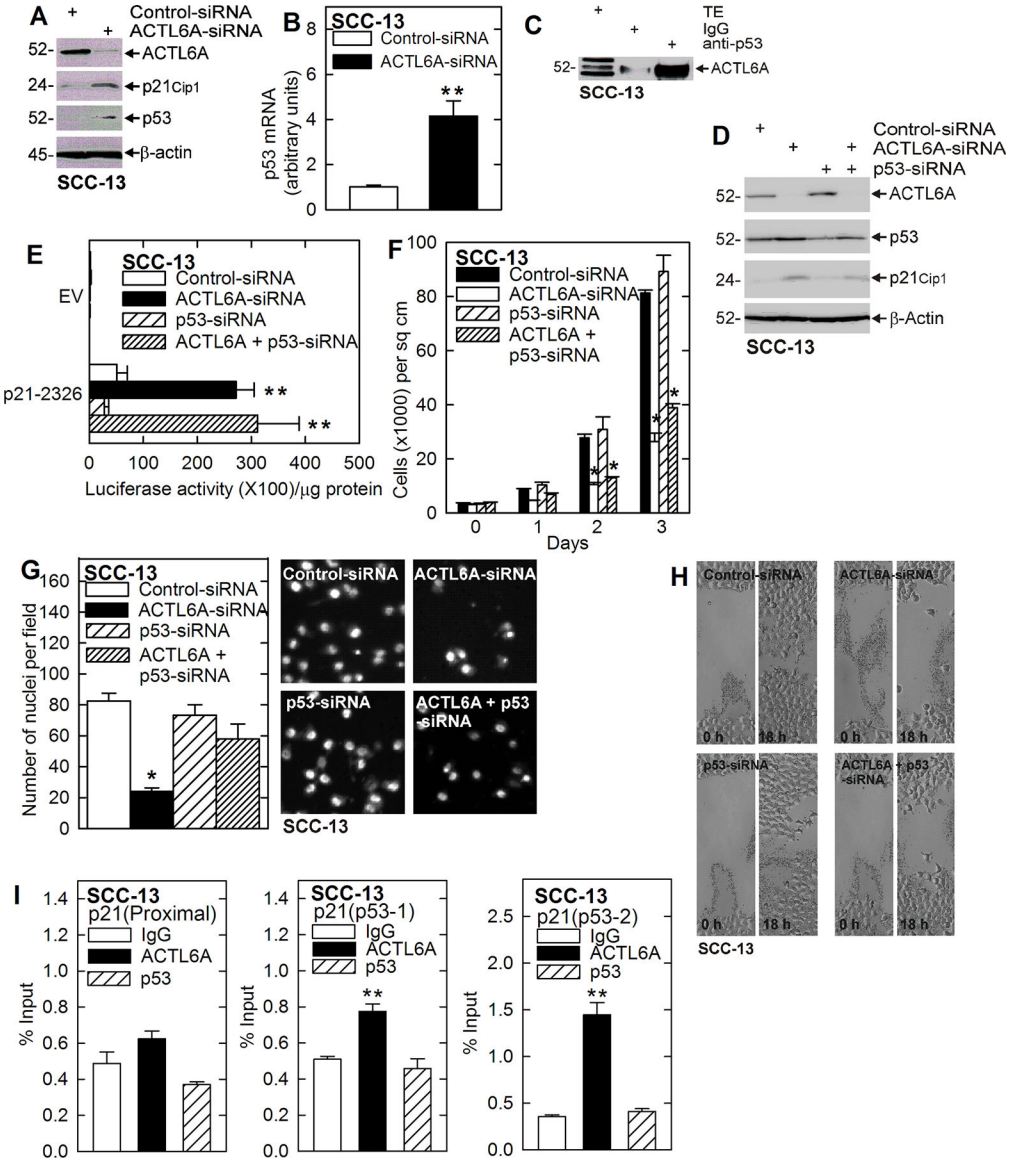

Fig. 3.

p53 is not required for ACTL6A regulation of $p21^{Cip1}$ level **A/B** ACTL6A-knockdown results in increased p53 and $p21^{Cip1}$ levels. **C** p53/ACTL6A interaction is documented by co-precipitation following incubation of 200 μg cell lysate with 1 μg IgG or anti-p53 antibody and immunoblot with anti-ACTL6A. **D** ACTL6A knockdown increases $p21^{Cip1}$ level, and this regulation was not impacted by manipulating p53 level. **E** ACTL6A knockdown increases $p21^{Cip1}$ promoter activity but manipulating p53 level does not alter $p21^{Cip1}$ promoter activity. **F/G/H** ACTL6A knockdown attenuates SCC-13 cell proliferation, invasion and migration, but these endpoints are minimally impacted by p53. **I** ChIP assay shows that ACTL6A, but not p53, interacts with the $p21^{Cip1}$ proximal promoter Sp1 region and the p53 response elements.

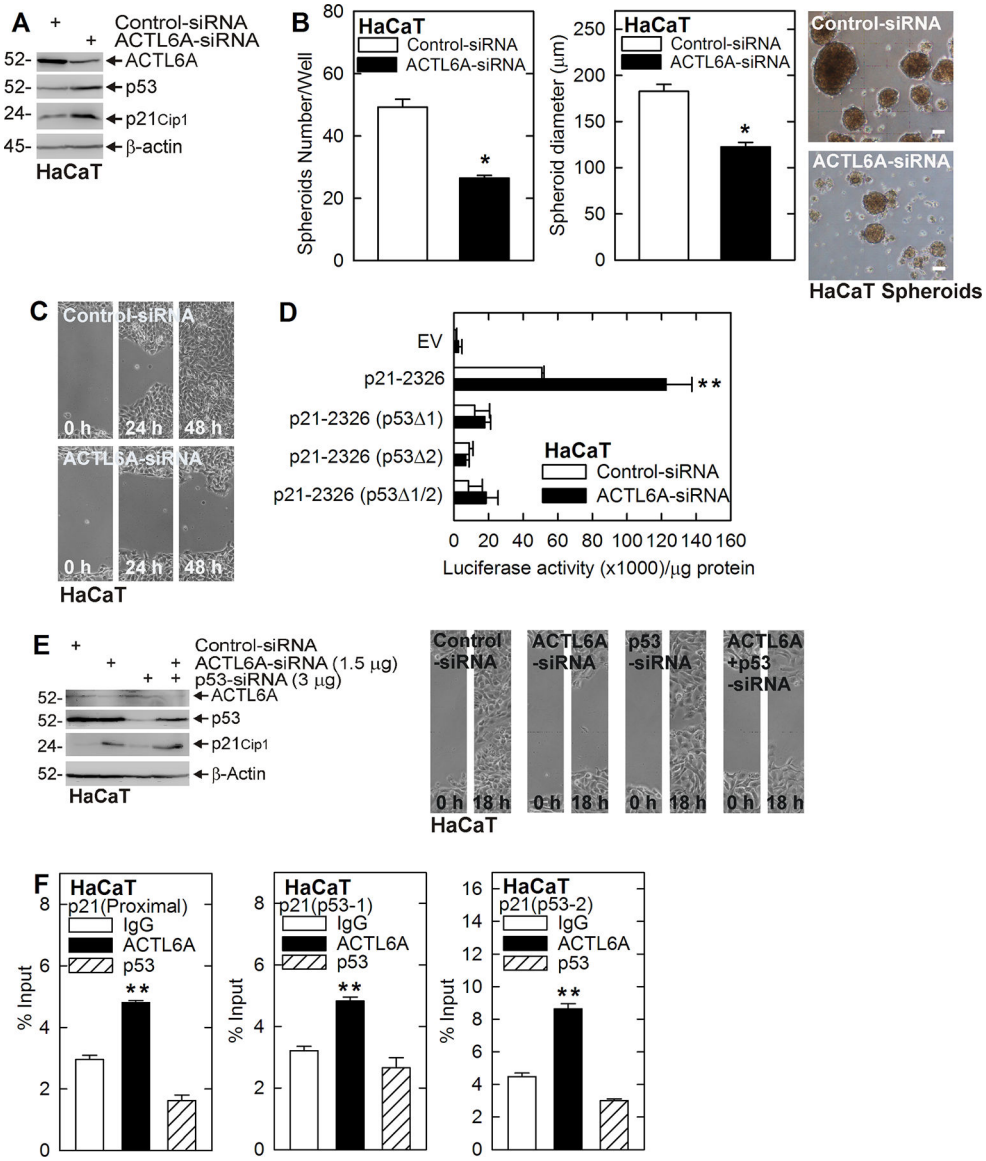

Fig. 4.

ACTL6A modulates p21^{Cip1} level to control the HaCaT phenotype **A** ACTL6A knockdown results in increased p53 and p21^{Cip1} levels. **B/C** ACTL6A knockdown reduces HaCaT cell spheroid formation and migration. **D** ACTL6A knockdown results in increased p21^{Cip1} promoter activity and this increase requires both p53 binding elements (p53-1 and p53-2). **E/F** ACTL6A knockdown increases p21^{Cip1} level, but p53 knockdown does not influence p21^{Cip1} level. ACTL6A loss reduces cell migration, but migration is minimally impacted by p53 knockdown. **G** ChIP analysis shows that ACTL6A interacts at the proximal promoter Sp1 region and the p53 binding sites (p53-1 and p53-2), but that p53 does not bind at these sites.

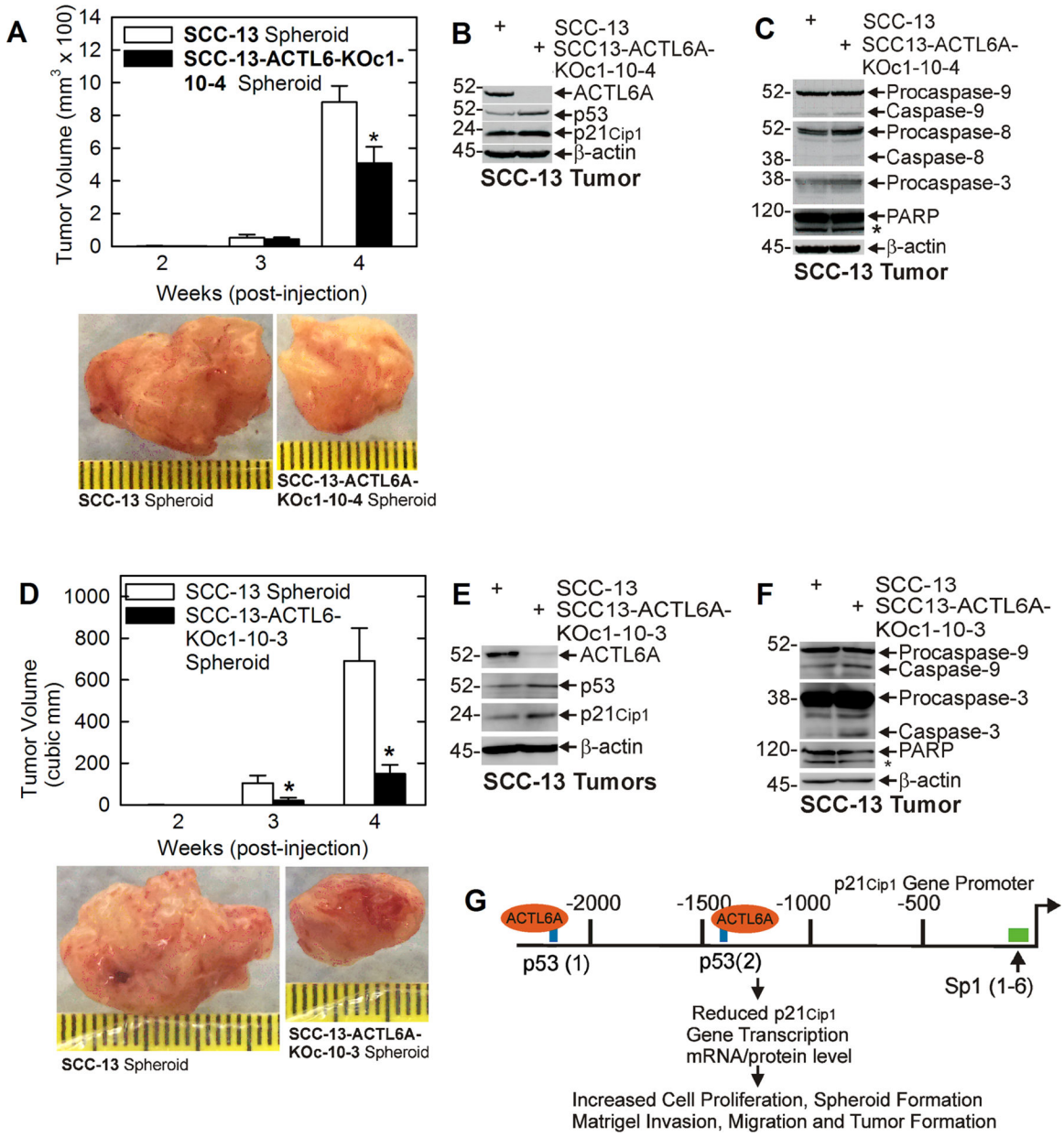

Fig. 5.

ACTL6A knockout reduces tumor formation **A** Spheroid-derived SCC-13 wild-type cells and SCC-13 ACTL6A knockout cell clones (SCC13-ACTL6A-KOc1-10-4 and SCC13-ACTL6A-KOc1-10-3) were injected into each front flank in NSG mice and tumor formation was monitored for 0 - 4 wk. **A/B/C** ACTL6A knockout cell (SCC13-ACTL6A-KOc1-10-4) tumor formation is reduces and this is associated with increased p53 and $p21^{Cip1}$ levels and minimal impact on apoptosis. **D/E/F** ACTL6A knockout cell (SCC13-ACTL6A-KOc1-10-3) tumor formation is reduces and this is associated with increased p53 and $p21^{Cip1}$ levels and activation of apoptosis. **G** Schematic describing the role of ACTL6A in regulating cancer cell function (details in text).