

FOSL1 Promotes Kras-induced Lung Cancer through Amphiregulin and Cell Survival Gene Regulation

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Abstract

The FOSL1/AP-1 transcription factor regulates gene expression, thereby controlling various pathophysiological processes. It is a major effector of RAS-ERK1/2 signaling and is activated in human lung epithelia by tumorigenic stimuli. Recent evidence shows an inverse correlation between *FOSL1* expression and the survival of patients with lung cancer and adenocarcinomas; however, its role in lung tumorigenesis remains elusive. In this work, we sought to determine the role of *FOSL1* in Kras-induced lung adenocarcinoma *in vivo* and its downstream effector mechanisms. We used mice expressing the Kras oncogene in the lung with concomitant *Fosl1* deletion, Kras-activated murine alveolar epithelial cells (mAECs) with *Fosl1* deletion, and KRAS mutant human lung adenocarcinoma (HLAC) cells with *FOSL1* deficiency, and performed cell proliferation and gene expression analyses. Mutant *Kras* induced *Fosl1* expression *in vitro* (mAECs) and *in vivo* (lung tissue), and mice with *Fosl1* deletion showed reduced levels of mutant *Kras*-induced lung tumorigenesis and survived longer than *Fosl1*-sufficient mice. Studies with mutant *Kras*-activated mAECs and KRAS-mutant HLAC cells revealed that *FOSL1* regulates mutant KRAS-induced gene expression, thereby controlling cell proliferation and survival. In contrast, *FOSL1* depletion in non-KRAS-mutant HLAC cells and nonmalignant human lung epithelia had no effect. Our data support the notion that FOSL1-mediated expression of amphiregulin

and apoptotic and antioxidative genes plays a role in regulating HLAC cell proliferation and survival. FOSL1 is a determinant of lung cancer *in vivo* and regulates HLAC cell proliferation and survival, largely in the context of KRAS mutations. Activation of *FOSL1* in adenocarcinomas may be a prognostic marker and potential target for human lung cancer with KRAS mutations.

Keywords: AP-1; apoptosis; cyclins; epidermal growth factors; tumor cell growth

Clinical Relevance

Mutant KRAS activation leading to lung tumor growth is a major cause of lung cancer deaths, but thus far its crucial downstream targets and mechanisms that control tumor cell growth are not well defined. Here, we show for the first time that FOSL1 is a determinant of lung cancer *in vivo* and regulates human lung adenocarcinoma cell proliferation and survival, largely in the context of KRAS mutations. Thus, activation of FOSL1 in adenocarcinomas may be a prognostic marker and potential target for human lung cancer with KRAS mutations.

Lung cancer is a prominent cause of cancer deaths in the United States and around the world. Alveolar epithelial cells (AECs) are major progenitors of lung cancer and are

direct targets of procarcinogenic agents. Members of the AP-1 family regulate the expression of genes that are critical for various pathophysiological processes (1)

that are also an integral part of various lung diseases and malignancy. Accumulating evidence suggests an important role for FOSL1 (aka FRA-1), a member of the FOS

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family, in cancer cell progression and maintenance of the transformed state in several cell types (2). Recent studies have shown that the FOSL1/AP-1 transcription factor regulates tumor heterogeneity, and especially tumor cell clonal evolution and epithelial and mesenchymal plasticity (3). Various procarcinogens, such as tobacco smoke (4), NNK (5), and tumor-promoting stimuli (6, 7), activate *FOSL1* expression in lung epithelia. *FOSL1* induction is required for asbestos-induced malignant transformation of rat pleural mesothelial cells, and *FOSL1* silencing reverses the malignant phenotype of mesothelioma *in vitro* (5, 8). The transition from the small cell to non-small cell lung cancer phenotype is accompanied by specific induction of *FOSL1*, suggesting that this transcription factor maintains the differentiated state of lung cancer cell types (9). FOSL1 is also expressed in other cancer cell types, including breast cancer (10), colon cancer (11), and hepatocellular carcinoma (12), suggesting an important role for this AP-1 transcription factor in both *Kras*- and non-*Kras*-driven tumorigenesis and progression (see reviews in References 3 and 13).

We previously demonstrated an obligatory role for metalloproteinase-EGF receptor (EGFR)-mediated, RAS-activated MAP kinase signaling in controlling smoke-induced *FOSL1* expression in lung epithelial cells (4). Ectopic *FOSL1* enhances lung epithelial cell motility and invasion, and causes anchorage-independent growth (14). Although the above data suggest a role for FOSL1 in mediating tumor epithelial cell progression *in vitro*, we found that *FOSL1* overexpression in nonmalignant lung epithelia alone is not sufficient to induce significant tumor growth in athymic mice (14). This suggests that additional potential modifications (such as phosphorylation) and/or the presence of some other activated proto-oncogene(s) are required to impart the full oncogenic potential of *FOSL1* *in vivo*.

Consistent with the above experimental data, emerging evidence shows a correlation between elevated levels of *FOSL1* expression and poor survival of patients with lung cancer and adenocarcinomas. However, whether FOSL1 is a critical determinant in driving the development and progression of lung cancer *in vivo*

remains unclear. In the present study, we examined for the first time the role of FOSL1 in regulating KRAS-induced human lung tumorigenesis using an experimental model of lung cancer. Here, we report that FOSL1 is required for mutant *Kras*-induced lung tumorigenesis *in vivo*, and promotes human lung adenocarcinoma (HLAC) growth and survival by regulating multiple gene networks that control cell proliferation and survival in the setting of KRAS mutations.

Methods

Mice

Mice bearing “floxed” *Fos1* alleles (15) were bred with mice with LSL-*Kras*^{G12D} (16) to generate bitransgenic *Fos1*^{F/F};*Kras*^{G12D} mice. Both *Fos1*^{F/F};*Kras*^{G12D} mice and *Kras*^{G12D} mice (8–10 weeks old, male and female) were infected with adenoviral vectors encoding *Cre* to activate *Kras* and concurrently delete *Fos1*. The animal protocols were approved by the Johns Hopkins Bloomberg School of Public Health and the University of Illinois at Chicago. More details are provided in the data supplement.

Cell Culture, Small Interfering RNA Transfection, Cell Proliferation, and Clonogenicity Assays

Details regarding cell culture, small interfering RNA (siRNA) transfection, cell proliferation, and clonogenicity assays are provided in the data supplement.

Histological, Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling, and Gene Expression Analyses

Details regarding histological, TUNEL, and gene expression analyses are provided in the data supplement.

Statistical Analysis

All histological and survival data were analyzed using GraphPad Prism4 software. Data are expressed as mean ± SD unless otherwise indicated. The survival data were also analyzed using a Kaplan–Meier survival plot. A two-tailed Student’s *t* test was used to calculate significance, and *P* = 0.05 or less was considered significant. Outliers identified by Grubbs’s test using the GraphPad calculator were removed from the data analysis.

Results

FOSL1 Overexpression Correlates Inversely with the Survival of Patients with Lung Cancer and Is Overexpressed in HLAC Cells

We assessed the relationship between *FOSL1* expression (Affymetrix ID 204420_at) and the survival of patients with lung cancer using the Kaplan–Meier Plotter (<http://kmpplot.com/analysis/>; for more details, see the data supplement). *FOSL1* mRNA expression and patient survival curves were plotted for all patients with lung cancer (*n* = 1,926) (Figure 1A, left panel), and also stratified for adenocarcinoma samples (*n* = 720) (Figure 1A, middle panel). High-level *FOSL1* mRNA expression was associated with poor overall survival rates in all patients with lung cancer. In contrast, relatively low-level *FOSL1* mRNA expression was associated with increased survival in all patients with lung cancer (*P* = 1.9e-09). Interestingly, increased *FOSL1* expression was associated with poor survival of patients with lung cancer and adenocarcinoma (*P* = 1.7e-07), but not patients with squamous cell carcinoma (*n* = 524, Figure 1A, right panel).

To verify the above findings, we examined *FOSL1* mRNA expression in four HLAC cell lines: A549, H1993, H2009, and H1437. Consistent with the data from the patients with lung cancer, the analysis revealed elevated levels of *FOSL1* mRNA expression in A549, H1993, H2009, and H1437 cells (Figure 1B). Corroborating the qRT-PCR results, immunoblot analysis showed an increased level of *FOSL1* expression, which was associated with its activation (phosphorylation, pFOSL1) status (Figure 1C). We next analyzed *FOSL1* expression in primary cultured normal human bronchial epithelial (NHBE) cells, and immortalized and nonmalignant human bronchial epithelial cell line (BEAS-2B) and human bronchial epithelial cell line (HBEC1). Immunoblot analysis revealed lower levels of FOSL1 and pFOSL1 in NHBE, BEAS-2B, and HBEC1 cells than in HLAC H1993 cells (Figure 1D).

Signaling by ERK1/2, not AKT1/2, Controls *FOSL1* Expression in HLAC Cells

Previously, we have shown that signaling by both ERK1/2 and AKT1/2 regulates *FOSL1* induction via tumor-promoting stimuli (e.g., phorbol ester and cigarette smoke) in human

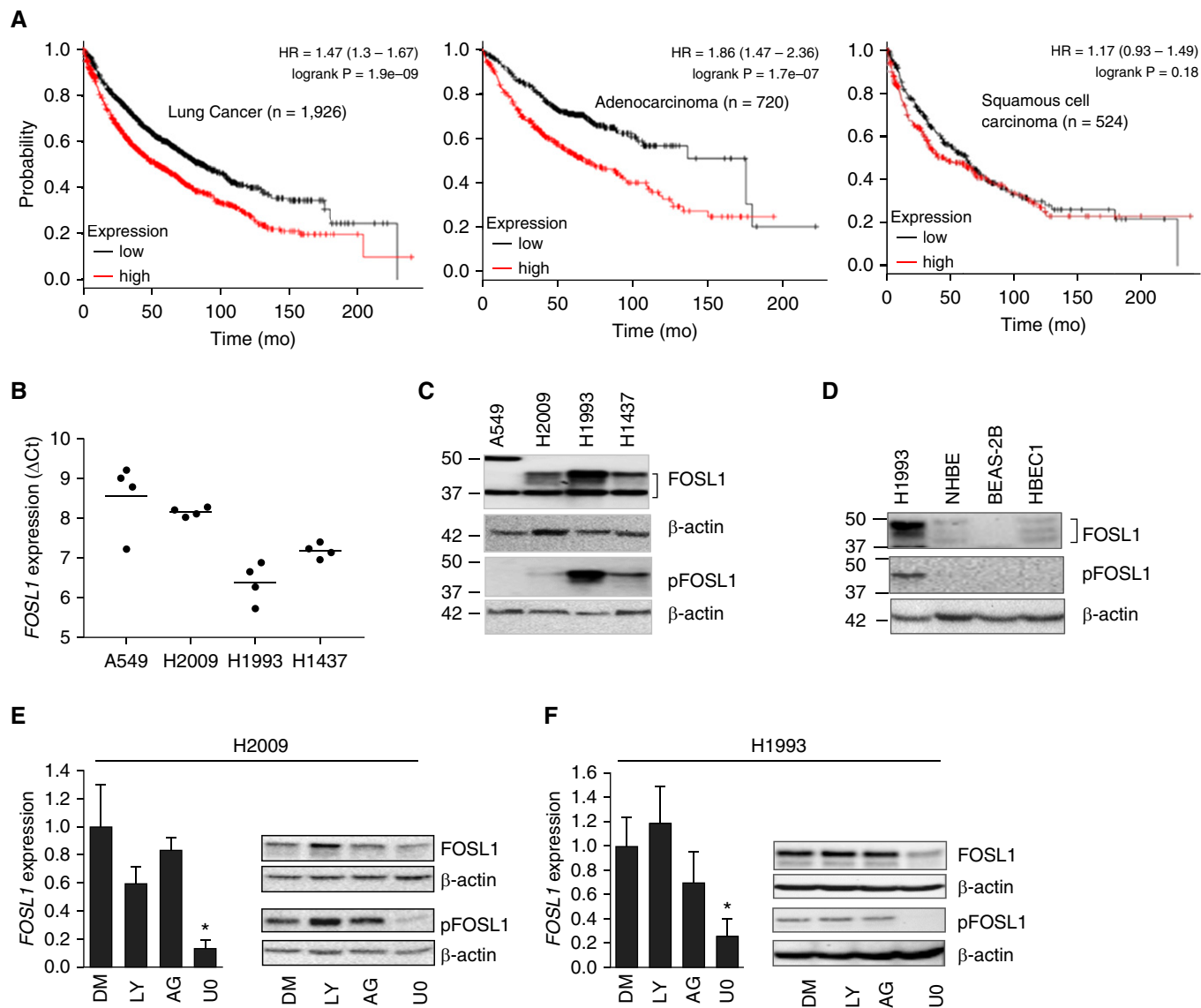


Figure 1. *FOSL1* expression in human lung cancer and its relationship with survival rates in patients with lung cancer. (A) *FOSL1* (Affymetrix ID 204420_at) expression and Kaplan–Meier plot of cumulative survival rates for all patients with lung cancer ($n = 1,926$; left), patients with lung adenocarcinoma ($n = 720$; middle), and patients with squamous cell carcinoma ($n = 524$; right). HR = hazard ratio. (B) *FOSL1* expression and regulation in human lung adenocarcinoma (HLAC) cells. *FOSL1* mRNA levels were quantified by qRT-PCR using β -actin as a reference. Values shown are Δ Ct. (C and D) Immunoblot analysis of the levels of native *FOSL1* and the activated (phosphorylated) form of *FOSL1* (p*FOSL1*) in (C) HLAC cells and (D) nonmalignant human lung epithelial cells. H1993 was used to compare the relative expression levels. (E and F) Effects of EGFR, PI3K-AKT1/2, and MEK1/2-ERK1/2 pathway inhibition on *FOSL1* expression in HLAC cells. H2009 and H1993 cells were treated with UO126 (UO, 10 μ M), LY294002 (LY, 10 μ M), AG1478 (AG, 10 μ M), or DMSO (DM, vehicle) for 6 hours, RNA was isolated, and *FOSL1* mRNA expression was analyzed by qRT-PCR. Values are expressed relative to control (DMSO). * $P \leq 0.05$, compared with DMSO. (E and F) Western blot showing *FOSL1* and p*FOSL1* levels in H2009 and H1993 cells treated with inhibitors as indicated. Readers may view the uncut gels and comments on the antibody probing protocol for Figure 1 in the data supplement. HBEC = human bronchial epithelial cell line; NHBE = normal human bronchial epithelial cells.

lung epithelial cells (4, 6, 7). To determine whether ERK1/2 and/or AKT1/2 signaling regulates *FOSL1* expression in human lung cancer, we treated H2009 (Figure 1E) and H1993 (Figure 1F) cells with UO126 (MEK1/2-ERK1/2 inhibitor), LY294002 (PI3K/AKT inhibitor), and AG1478 (EGFR inhibitor) for 6 hours, and analyzed *FOSL1* (mRNA and

protein) expression. Both qRT-PCR and immunoblot analyses revealed decreased levels of *FOSL1* expression in H2009 and H1993 cells treated with UO126 (Figures 1E and 1F), whereas LY294002 and AG1478 had no such effect, demonstrating that *FOSL1* expression in HLAC cells is largely regulated by MEK1/2-ERK1/2 signaling.

Deletion of *Fos1* Decreases Mutant *Kras*-induced Lung Tumor Growth and Promotes Survival

We first assessed *Fos1* activation by mutant *Kras* *in vivo* in the lung and alveolar epithelia. We found an increased expression of *Fos1* mRNA in the lungs of mice and in primary type 2 AECs (AEC2s) with mutant *Kras*

activation (Figure E1 in the data supplement), demonstrating that mutant *Kras* induces *Fos1* expression in lung epithelia. To define the role of FOSL1 in human lung tumorigenesis *in vivo*, we generated a bitransgenic mouse model bearing *Fos1* “floxed” alleles and LSL-*Kras*^{G12D} (notated as *Fos1*^{F/F};*Kras*^{G12D}). *Fos1*^{F/F};*Kras*^{G12D} mice were infected with adenoviral Cre to express oncogenic *Kras* and concomitantly delete *Fos1* in the lung (Figure 2A). Mice were killed at 13 weeks and 20 weeks after infection to monitor lung tumor development

and progression. The survival rates of these mice after tumor initiation were also monitored. There was a significant increase in the median survival rate of *Fos1*^{F/F};*Kras*^{G12D} mice as compared with *Kras*^{G12D} mice (Figure 2B). This result suggests an important role for *Fos1* in mediating oncogenic-*Kras*-induced lung tumorigenesis.

Consistent with published reports, the *Kras*^{G12D} mice displayed visibly distinct adenomas on both lobes of the lungs at 13 weeks after tumor initiation (Figure 2C, top panel). However, fewer or no adenomas

were visible on the lungs of *Fos1*^{F/F};*Kras*^{G12D} mice. At 20 weeks after *Kras* activation, large adenocarcinomas were visible in *Kras*^{G12D} mice, but there were fewer and smaller adenocarcinomas in *Fos1*^{F/F};*Kras*^{G12D} mice (Figure 2C, bottom panel). These differences in the rate of development and the size of adenomas/adenocarcinomas were clearly visible in hematoxylin and eosin-stained lung sections obtained from both groups of mice at the respective time points (Figure 2D). Consistent with these observations, the tumor area in *Kras*^{G12D} mice at 13 weeks and

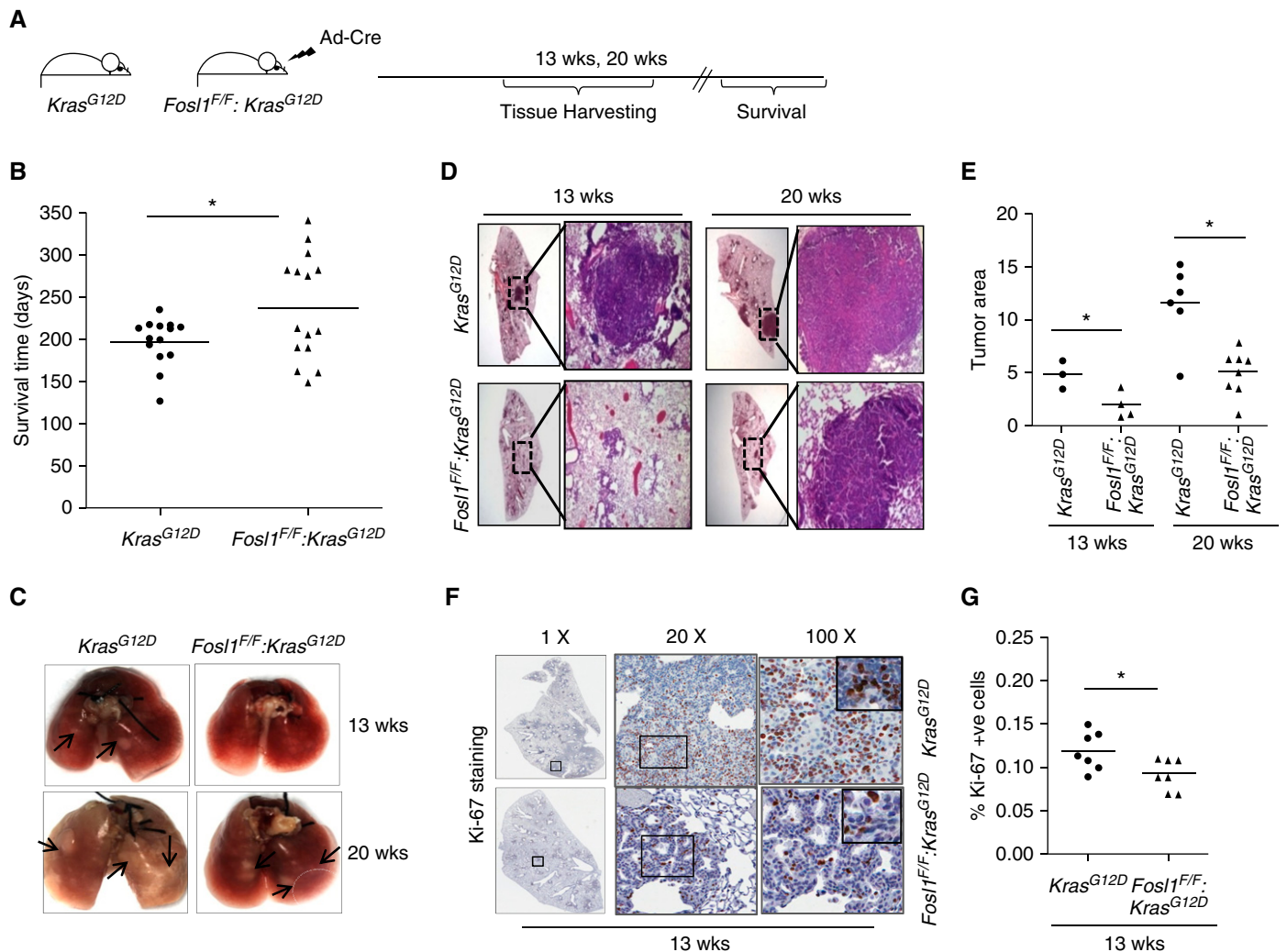


Figure 2. Disruption of *Fos1* reduces mutant *Kras*-induced lung tumorigenesis *in vivo*. (A) Schema showing the timeline of tissue harvesting and survival monitoring of *Kras*^{G12D} mice and *Fos1*^{F/F};*Kras*^{G12D} mice after adenoviral Cre vector infection. (B) Survival rates of *Kras*^{G12D} mice and *Fos1*^{F/F};*Kras*^{G12D} mice after mutant *Kras* activation. **P* ≤ 0.05, *Kras*^{G12D} versus *Fos1*^{F/F};*Kras*^{G12D} mice. (C) Representative lungs harvested from *Kras*^{G12D} mice and *Fos1*^{F/F};*Kras*^{G12D} mice at 13 weeks and 20 weeks after infection. Arrows indicate the position of adenomas. (D) Representative photomicrographs of hematoxylin and eosin-stained lung tumor sections at 13 weeks and 20 weeks after mutant *Kras* activation. Low (×1, left) and high (×20, right) magnifications are shown. (E) Quantification of tumor area (percent) in *Kras*^{G12D} mice and *Fos1*^{F/F};*Kras*^{G12D} mice. Values are mean ± SD. **P* ≤ 0.05, versus *Kras*^{G12D} mice. (F) *Fos1* deletion affects *Kras*-induced tumor cell proliferation. Lung tumor sections at 13 weeks of *Kras* activation were immunostained with Ki-67 antibody. Shown are representative photomicrographs at low (×1, left) intermediate (×20, middle), and high (×100, right) resolution. (G) Quantification of Ki-67⁺ cells in lungs of *Kras*^{G12D} mice and *Fos1*^{F/F};*Kras*^{G12D} mice. **P* ≤ 0.05, versus *Kras*^{G12D} mice.

20 weeks was quantified as 4.9% and 12%, respectively. However, it was significantly smaller in their *Fos1^{F/F}:Kras^{G12D}* counterparts, which had an average lung tumor area of 2% and 5.1% at 13 weeks and 20 weeks, respectively (Figure 2E). To determine whether the reduced tumor burden in *Fos1^{F/F}:Kras^{G12D}* mice was accompanied by a reduction in tumor cell proliferation, we performed Ki-67 immunostaining of the lungs at 13 weeks after tumor initiation (Figure 2F). The number of proliferating (Ki-67⁺) cells in the transformed areas of lung sections from *Fos1^{F/F}:Kras^{G12D}* mice was significantly lower than that observed for *Kras^{G12D}* mice (Figure 2G). Collectively, these data suggest that the

Fos1/AP-1 transcription factor is important for mutant *Kras*-induced lung tumor cell proliferation and tumor growth *in vivo*.

Fos1 Selectively Upregulates Mutant Kras-Induced Cell-Cycle Gene Expression in Alveolar Epithelia

To examine the mechanisms underlying the *Fos1*-mediated decrease in *Kras*-induced lung epithelial cell proliferation, AEC2s were isolated from lungs of *Kras^{G12D}* mice and *Fos1^{F/F}:Kras^{G12D}* mice, cultured for 4 days, and then infected with adenoviral vectors expressing Cre to activate mutant *Kras* (outlined in Figure 3A). Cells were infected with adenoviral vectors expressing

green fluorescent protein to serve as a control. At 48 hours and 96 hours after infection, RNA was isolated from AEC2s for gene expression analysis. As anticipated, an ~3-fold increased expression of *Fos1* mRNA was found in *Kras^{G12D}* AEC2s with *Kras* activation. *Fos1* expression was markedly (~80%) lower in *Kras*-activated *Fos1^{F/F}:Kras^{G12D}* AEC2s at both 48 hours and 96 hours after infection, demonstrating adequate deletion of *Fos1* in the AEC2s (Figure 3B). To further determine the role of *Fos1* in regulating *Kras*-induced cell proliferation, we analyzed the expression levels of several genes encoding cyclins and growth factors involved in cell-cycle

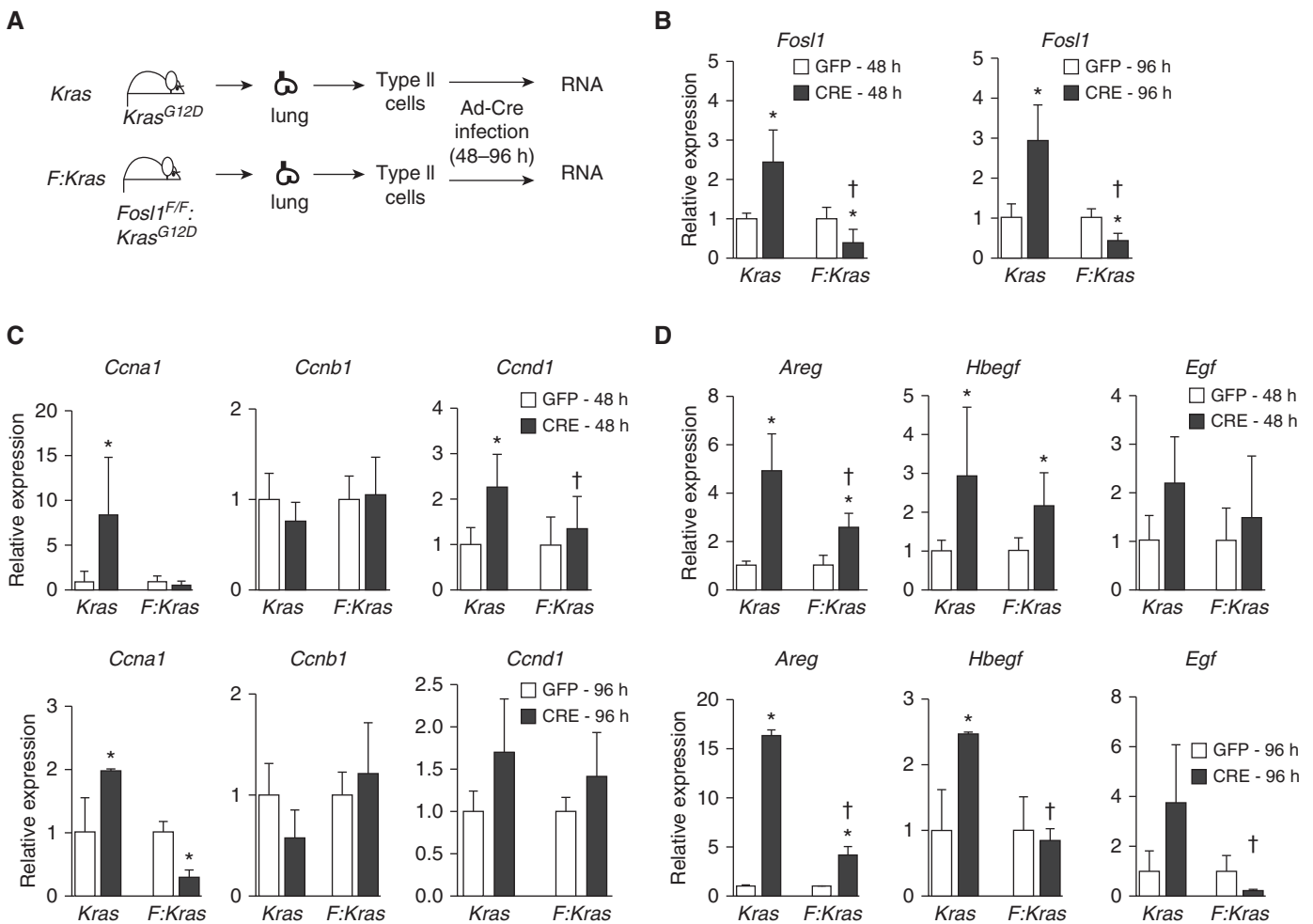


Figure 3. Mutant *Kras*-induced cell-cycle gene expression in primary alveolar epithelia with and without *Fos1* deletion. (A) Type 2 alveolar epithelial cells (AEC2s) were isolated from lungs of *Kras^{G12D}* (*Kras*) mice and *Fos1^{F/F}:Kras^{G12D}* (*Fos1:Kras*) mice. *Kras* AEC2s and *Fos1:Kras* AEC2s (abbreviated as *Kras* and *F:Kras*, respectively) were cultured for 4 days and infected with adenovirus vectors encoding Cre or GFP for 48 hours or 96 hours. RNA was isolated and gene expression was analyzed by qRT-PCR using β -actin as a reference. (B) *Fos1* mRNA expression in AEC2s of the indicated genotypes at 48 hours and 96 hours after adenoviral-Cre/GFP infection. (C) Analysis of cell-cycle progression and gene expression at 48 hours (top) and 96 hours (bottom) after infection. (D) Expression levels of EGFR ligands in AEC2s of the *Kras* and *Fos1:Kras* genotypes at 48 hours (left panel) and 96 hours (bottom) after infection. Values are expressed relative to the respective genotypes infected with adenoviral GFP. * $P \leq 0.05$, Cre versus GFP; † $P \leq 0.05$, versus *Kras*.

progression. Increased expression of *Ccna1* and *Ccnd1* was noted in *Kras*^{G12D} AEC2s expressing mutant *Kras* at both 48 hours (Figure 3C, top) and 96 hours (Figure 3C, bottom) after infection, but their expression was not significantly induced in *Fosl1*^{F/F};*Kras*^{G12D} AEC2s with *Fosl1* deletion. *Ccnb1* mRNA expression was not altered in AEC2s of either genotype with *Kras* activation, suggesting selective upregulation of cyclins by mutant *Kras* in lung epithelia. As increased expression of various growth factors that regulate EGFR activation is important for cancer cell growth and survival (17), we examined the expression of the EGFR ligands *Areg*, *Hbegf*, and *Egf* in *Kras*-activated AEC2s without and with *Fosl1* deletion. *Areg* and *Hbegf* were significantly upregulated by mutant *Kras* in *Kras*^{G12D} AEC2s, but they showed significantly lower induction in *Kras*^{G12D} AEC2s lacking *Fosl1* (Figure 3D). These data indicate that the *Fosl1* transcription factor is required for upregulating mutant *Kras*-induced gene expression involved in AEC proliferation and survival.

FOSL1 Is Required for the Growth of HLAC Cells with Mutant KRAS

To determine whether FOSL1 is required for the promotion of KRAS mutant HLAC cell proliferation and survival, we used H2009 cells expressing elevated levels of FOSL1 (Figure 1C) and harboring the KRAS mutation. We used custom *FOSL1* siRNA that was previously shown to be effective and specific for depleting *FOSL1* (18, 19) (Figure E2). *FOSL1* knockdown using the siRNA-mediated approach significantly reduced proliferation of H2009 cells (Figure 4A) and markedly impaired their survival, as assessed by long-term clonogenicity assays (Figure 4B). Similar results were obtained using smart pooled *FOSL1*-specific siRNAs (Figure E3). We used custom *FOSL1* siRNA for the rest of studies. *FOSL1* silencing also reduced KRAS mutant H2030 (Figure E4) and A549 (Figure E5A) cell proliferation, but its knockdown had no antiproliferative effect in nonmalignant immortalized bronchial epithelial cells (BEAS-2B; Figure E5B). Likewise, *FOSL1* depletion had no antiproliferative effects in HLAC cells that harbored wild-type KRAS (H1993 and CL1-5), despite elevated expression of *FOSL1* in these cells (unpublished data). These results suggest that FOSL1 is largely important for mutant KRAS-regulated

HLAC cell growth and survival, but not for the survival of non-KRAS-mutant cells (H1993 and CL1-5) *in vitro*.

FOSL1 Maintains the Survival of KRAS Mutant HLAC Cells by Repressing Apoptosis through Modulation of Antioxidative and Apoptotic Gene Expression

To delineate the mechanisms by which FOSL1 promotes HLAC cell proliferation and survival, we assessed apoptosis in *FOSL1* siRNA-transfected KRAS mutant H2009 cells. TUNEL staining revealed the induction of apoptosis, as shown by increased levels of TUNEL-positive cells, in *FOSL1*-depleted cells (Figure 4C). *FOSL1* depletion resulted in reduced levels of antiapoptotic *BCL2* and *BCLXL* expression (Figure 4D), suggesting the modulation of apoptotic gene expression by FOSL1 in KRAS mutant HLAC cells. As optimal antioxidative gene expression is important for mitigating oxidative stress and maintaining tumor cell survival, we examined the effect of *FOSL1* silencing on their expression in H2009 cells (Figure 4D). *NQO1* and *HMOX1* expression was upregulated, whereas *GCLC* and *GCLM* expression was not altered due to *FOSL1* deficiency, suggesting that this transcription factor selectively modulates antioxidative gene expression and maintains HLAC cell survival.

Apoptosis is a complex process that is regulated by multiple proteins and pathways. Thus, to verify that FOSL1 regulates KRAS mutant HLAC cell survival through the modulation of apoptosis, H2009 cells with *FOSL1* knockdown were supplemented with ZVAD (a general inhibitor of apoptosis) and their proliferation and potential for clonogenicity were assessed (Figure 4E, top). ZVAD supplementation rescued cell growth defects observed in H2009 cells with the *FOSL1* knockdown. ZVAD modestly improved H2009 cell survival (Figure 4E, bottom), as assessed by long-term clonogenicity assays, imparted by *FOSL1* deficiency.

FOSL1 Promotes HLAC Cell Growth by Regulating AREG Expression

Our studies in primary cultured murine AEC2s suggest that FOSL1 upregulates the expression of growth factors that activate EGFR signaling induced by mutant *Kras*. Among EGFR ligands, *Areg* expression was

markedly upregulated in alveolar epithelia by the *Kras* oncogene in a *Fosl1*-dependent manner (Figure 3D). As AREG has been implicated in human cancer (20), we analyzed *Areg* expression in *Kras*-induced lung tumors with and without *Fosl1* deletion. *Kras*-induced lung tumors at 13 weeks showed increased levels of *Areg* expression in lungs of *Kras* mutant mice, but its expression was significantly lower in lung tumors obtained from mice with *Fosl1* deletion (Figure 5A). Likewise, *Areg* expression in *Kras*-induced lung tumors at 20 weeks in mice lacking *Fosl1* was lower than in mice without *Fosl1* deletion. To verify that FOSL1 regulates AREG expression in human lung cancer, we measured the latter expression in H2009 cells with *FOSL1* depletion. AREG mRNA expression in H2009 cells was reduced by *FOSL1* deficiency (Figure 5B), suggesting a potential role for AREG in mediating KRAS-induced HLAC cell growth. Indeed, AREG silencing decreased H2009 cell growth, as assessed by acute cell proliferation (Figure 5C) and long-term clonogenicity assays (Figure 5D). Supplementation of AREG in *FOSL1* siRNA-transfected H2009 cells improved their growth defects imparted by *FOSL1* deficiency (Figure 5E).

AREG silencing did not alter *HMOX1*, *GCLC*, or *GCLM* expression levels, although *NQO1* expression was modestly increased (Figure 6A). In contrast, AREG silencing significantly reduced the expression levels of *Cyclin D1*, but not *Cyclin A1* and *Cyclin B1* (Figure 6B). Likewise, *FOSL1* silencing significantly reduced the expression levels of *Cyclin D1*, but not other cyclins (Figure 6C). *FOSL1* silencing resulted in a modest increase in p21, cyclin dependent-kinase inhibitor 1 expression. In contrast, AREG depletion had no such effect on p21 expression. Collectively, these results suggest that AREG acts as a downstream effector of FOSL1 and mediates HLAC cell growth by selectively regulating FOSL1-dependent *Cyclin D1* expression, but not by modulating antioxidative gene expression.

Discussion

High-level *FOSL1* expression in HLAC is associated with poor survival of patients with lung cancer and adenocarcinoma.

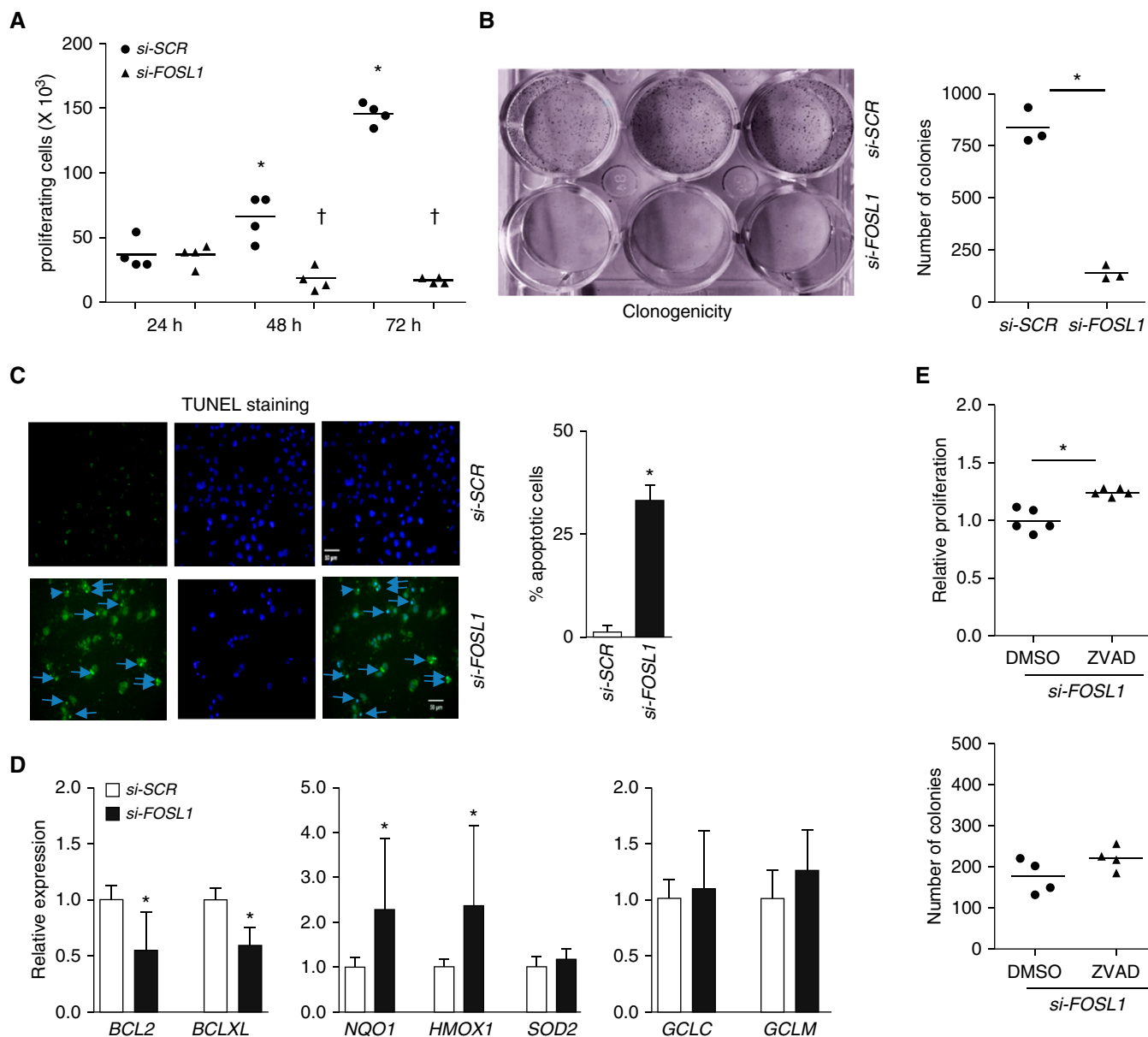


Figure 4. Effects of FOSL1 deficiency on KRAS mutant HLAC cell proliferation and survival. (A) To measure cell proliferation, H2009 cells (15,000/well) transfected with scramble (SCR) siRNA or custom *FOSL1* siRNA (25 nM) for 24 hours were plated and harvested at the indicated time points and counted. Data are mean \pm SD. * $P \leq 0.05$ versus 24 hours *si-SCR*; $^{\dagger}P \leq 0.05$, versus *si-SCR*. (B) To measure long-term survival, H2009 cells (1,500/well) transfected with SCR or *FOSL1* siRNA were seeded on a 12-well plate and cultured for 7 days. Colonies were stained with crystal violet, dried, photographed, and quantified. Data are mean \pm SD. * $P \leq 0.05$, versus *si-SCR*. (C) FOSL1 deficiency promotes apoptosis in KRAS mutant HLAC cells. To examine whether FOSL1 deficiency affects cell proliferation by inducing apoptosis, H2009 cells (15,000/well) were transfected with *si-SCR* or custom *si-FOSL1* siRNA (25 nM) for 72 hours and stained with TUNEL and DAPI. Images were then captured and TUNEL-positive cells (see arrows) were enumerated. Merged images stained with TUNEL and DAPI for 72 hours are shown. Data are mean \pm SD ($n = 3$); * $P \leq 0.05$ versus *si-SCR*. Scale bars: 50 μ m. (D) H2009 cells were transfected with *si-SCR* or *si-FOSL1* for 72 hours, RNA was isolated, and antiapoptotic and antioxidative gene expression (as indicated) was analyzed. Values are shown relative to *si-SCR*. Data are mean \pm SD. * $P \leq 0.05$, versus *si-SCR*. (E) To determine the role of apoptosis, H2009 cells transfected with *FOSL1* siRNA were seeded on a 12-well plate and then cultured in the presence of ZVAD or vehicle (DMSO) for 3 days to measure their proliferation rate (top) or 7 days to measure their clonogenicity (bottom) as in Figure 6. Data are mean \pm SD. * $P \leq 0.05$, versus DMSO.

Using a mouse model of lung cancer, we show that *Fosl1* deletion results in increased survival of mice with mutant *Kras*-induced lung tumorigenesis, demonstrating an

important role for this transcription factor in promoting mutant *KRAS*-induced lung cancer *in vivo*. Furthermore, our data reveal that by upregulating AREG expression and

modulating antiapoptotic and antioxidative gene expression, FOSL1 maintains oncogenic *KRAS*-induced lung cancer cell growth and survival.

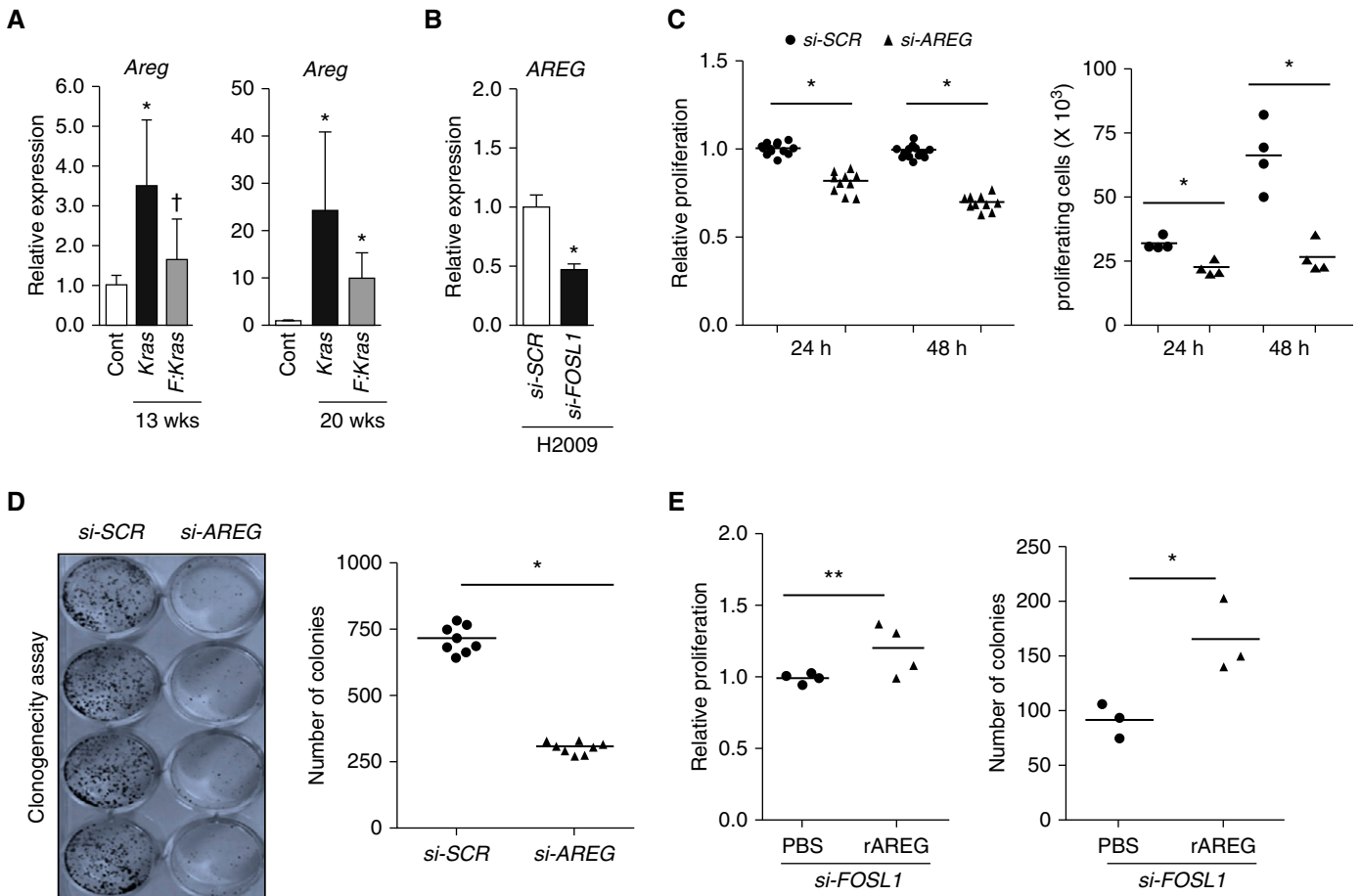


Figure 5. FOSL1 deficiency dampens mutant *Kras*-induced AREG expression in lung tumors, and FOSL1 promotes KRAS mutant HLAC cell proliferation by regulating AREG expression. (A) *Areg* mRNA expression in lung tumors of *Kras*^{G12D} mice and *Fos1*^{F/F};*Kras*^{G12D} mice at 13 weeks and 20 weeks after *Kras* oncogene activation. Values are expressed relative to the lung tissue of *Kras*^{G12D} mice (notated as Cont) without adenoviral Cre infection. Data are mean \pm SD ($n = 3-6$). * $P \leq 0.05$ versus Cont; † $P \leq 0.05$ versus *Kras*. (B) *AREG* expression in H2009 cells transfected with *si-SCR* or *si-FOSL1* for 72 hours. Data are mean \pm SD ($n = 3-4$). * $P \leq 0.05$ versus *si-SCR*. (C) H2009 cells (15,000/well) transfected with *SCR* siRNA or *AREG* siRNA (100 nM) for 24 hours were seeded onto a 96-well plate. Cells were harvested at 24 hours and 48 hours thereafter, and their proliferation was measured by CellTiter-Glo assay (left) or cell counting (right). Data are mean \pm SD. * $P \leq 0.05$, versus *si-AREG*. (D) To measure clonogenicity, H2009 cells (1,500/well) transfected with *si-SCR* or *si-AREG* for 24 hours were seeded onto a 12-well plate and cultured for 7 days. Colonies were stained with crystal violet, dried, and photographed (left), and the number of colonies was enumerated (right). Data are mean \pm SD. * $P \leq 0.05$ versus *si-SCR*. (E) Exogenous AREG improves HLAC cell survival imparted by *FOSL1* deficiency. H2009 cells transfected with *FOSL1* siRNA were seeded on a 96-well or 12-well plate and then cultured in the presence of recombinant AREG (rAREG; 50 ng/ml) or vehicle (PBS) for 3 days to measure their proliferation rate (left) or 7 days to measure clonogenicity (right) as in Figure 4. Data are mean \pm SD. * $P \leq 0.05$, versus PBS. ** $P = 0.06$ versus PBS.

The FOSL1/AP-1 transcription factor has been shown to regulate KRAS mutant HLAC cell proliferation and survival (21), and is important for oncogenic KRAS-induced non-lung cancer cell proliferation and progression *in vitro* (2, 22). Our studies provide a novel insight into the role of FOSL1-mediated mechanisms in regulating lung cancer cell proliferation. Loss-of-function (siRNA-mediated knockdown) and gain-of-function (exogenous supplementation) studies identified AREG as a downstream effector of FOSL1 that is required for promoting KRAS mutant HLAC cell proliferation

(Figure 5). Consistent with this result, we observed decreased levels of AREG expression in the lung tumors of *Fos1*^{F/F};*Kras*^{G12D} mice as compared with their *Kras*^{G12D} counterparts (Figure 5A), and in oncogenic *Kras*-activated mouse lung alveolar epithelia with *Fos1* deletion (Figure 3D). These data suggest that by upregulating AREG expression in alveolar epithelia, FOSL1 promotes *Kras* oncogene-induced lung tumor cell proliferation and growth *in vivo*. The EGFR ligands EGF, TGF- α , HBEGF, and AREG are differentially expressed during development and inflammatory and

wound-healing responses, and are known to elicit distinct cellular responses (23). For example, EGF induces the epithelial-mesenchymal transition, whereas AREG promotes proliferative responses, suggesting that EGFR ligands have different effects on epithelial cell proliferation and differentiation. Several lines of evidence show that EGFR ligands provoke distinct cellular responses by differentially modulating EGFR degradation or recycling. For example, the binding of EGF and TGF- α largely promotes EGFR degradation. In contrast, AREG binding to EGFR facilitates its recycling, but not its

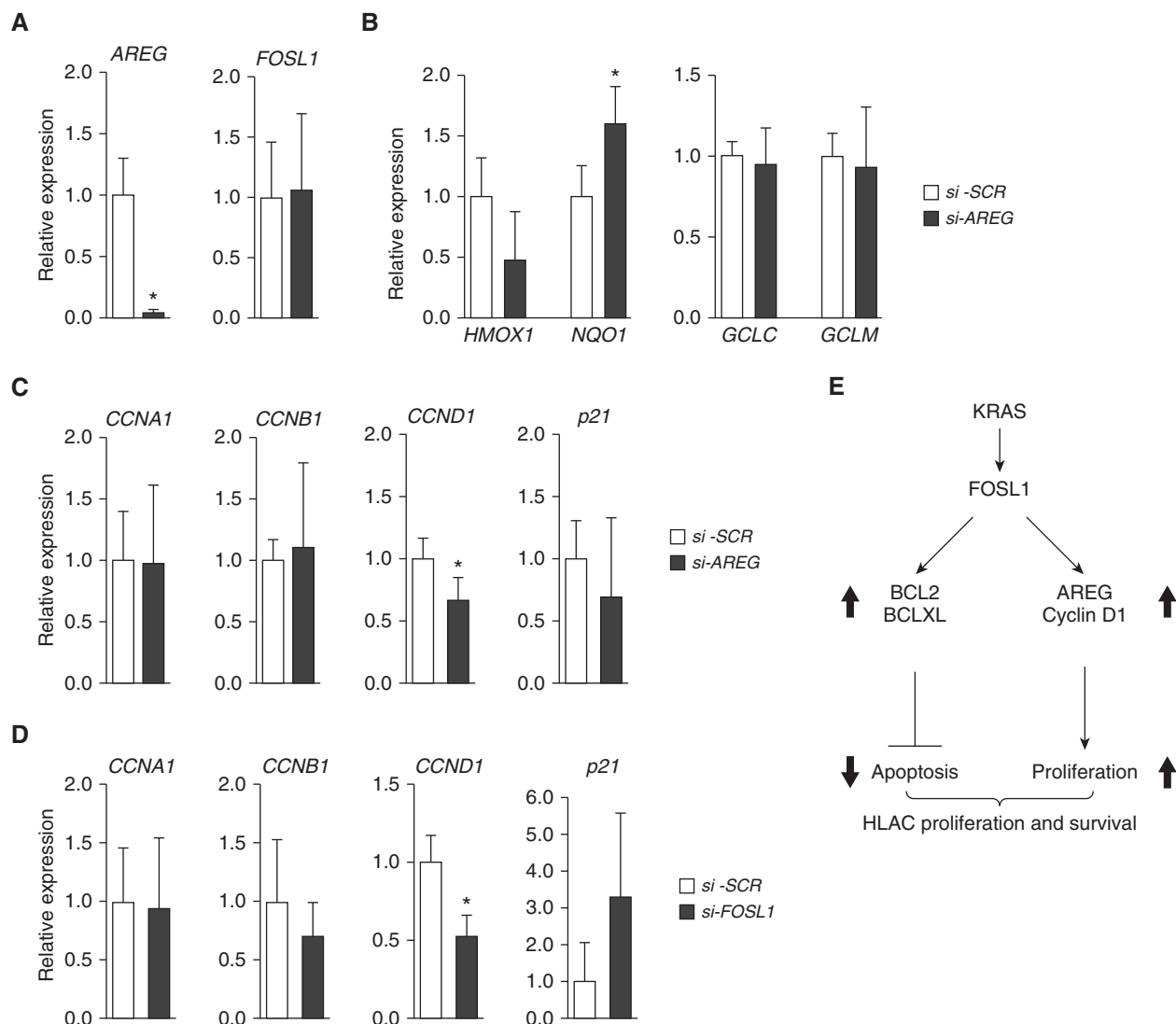


Figure 6. Effects of AREG deficiency on FOSL1 and FOSL1-regulated cell-cycle and antioxidative gene expression in HLAC cells. (A) *AREG* and *FOSL1* expression in H2009 cells transfected with *SCR* siRNA or *AREG* siRNA (100 nM) for 72 hours. (B) Analysis of antioxidative and (C) cell-cycle gene expression in H2009 cells transfected with *si-SCR* or *si-AREG*. The indicated gene expression was quantified by qRT-PCR analysis. (D) Analysis of cell-cycle gene expression in H2009 cells with *FOSL1* depletion. Data in A–D are mean \pm SD ($n = 3-4$). * $P \leq 0.05$ versus *si-SCR*. (E) *KRAS* oncogene-induced *FOSL1* activation promotes increased expression of *AREG*, cyclin D1, *BCL2*, and *BCLXL*. *BCL2* and *BCLXL* are required for cell survival, whereas *AREG* maintains cell proliferation by regulating cyclin D1 expression. Decreased levels of *AREG* expression due to lack of the *FOSL1* transcription factor result in impaired HLAC cell growth.

degradation, resulting in an increased level of EGFR-activated signaling (reviewed in Ref. 24). Although *AREG* plays a key role in tissue development and morphogenesis, several studies have shown an important role for *AREG* in cancer cell survival and progression (20). Recently, it was reported that *AREG* expression correlates with epithelial cell hyperplasia and the development of cigarette smoke-induced lesions in the human lung (25). AEC2 stem cell renewal during alveolar epithelium repair after lung injury is mediated by

EGFR ligand-activated signaling and by *Kras*-activated signaling during lung cancer development (26). Our results from *Fos1*-sufficient and *Fos1*-deficient *Kras*-induced lung tumors and lung alveolar epithelia, and *KRAS* mutant HLAC cells with *FOSL1* depletion reveal that *FOSL1* is important for the upregulation of *AREG* expression during *Kras*-induced lung tumorigenesis. *AREG* silencing results in decreased proliferation, and exogenous *AREG* rescues cell growth defects caused by *FOSL1* deficiency, implying that *AREG* serves as a

downstream effector of *FOSL1* and mediates HLAC cell proliferation. Note that consistent with our results, Vallejo and colleagues (27) recently reported that *Fos1* regulates mutant *Kras*-induced lung tumorigenesis through the expression of genes involved in cell-cycle progression (e.g., aurora kinase A). Via a cross-species meta-analysis of gene expression, they also identified *AREG*, in addition to *FOSL1*, as a top candidate gene in lung cancer (27). Nonetheless, our studies demonstrate for the first time a causal link between

FOSL1-regulated AREG expression and human lung cancer cell survival.

Increased proliferation and resistance to death are the essential hallmarks of the cancer cell (28). Here, we found that *FOSL1* silencing attenuated HLAC cell proliferation rates and promoted cell death, but had no effect on nonmalignant human lung epithelia (Figure E5), suggesting that FOSL1 is important for KRAS mutant HLAC cell proliferation and survival. Dysregulation of apoptotic and antiapoptotic gene expression has been implicated in lung cancer cell survival. The findings that blocking of apoptosis reversed the effects of FOSL1 deficiency on HLAC cell proliferation, and that HLAC cells have high levels of *FOSL1* expression (transcripts and protein) support our contention that FOSL1 maintains HLAC cell survival by inhibiting apoptosis. We have previously shown that mouse embryonic fibroblasts lacking *Fosl1* are resistant to oxidant stress, and this phenotype is associated with increased expression of NFE2L2 (aka Nrf2)-regulated antioxidant genes (*HMOX1*, *NQO1*, *GCLM*, and *GCLC*) (29). However, our studies with HLAC cells showed that *FOSL1* depletion increased expression of *HMOX1* and *NQO1*, but did not alter *GCLC* or *GCLM* expression (Figure 4D), suggesting that FOSL1 differentially regulates antioxidant gene expression in HLAC cells as compared with normal cells. Studies with the apoptosis inhibitor ZVAD showed that inhibition of apoptosis prevented the effects of *FOSL1* deficiency on HLAC cell survival, suggesting that the FOSL1 transcription factor maintains lung cancer cell survival by regulating apoptosis. The exact mechanisms by which FOSL1

modulates apoptosis in HLAC cells warrant a detailed investigation.

Our studies show that FOSL1 contributes to HLAC cell growth in an oncogenic mutation-dependent manner. Knockdown of *FOSL1* expression inhibited the proliferation of KRAS mutant HLAC cells (H2009, H2030, and A549), but did not affect non-KRAS-mutant cells (CL1-5 with a *p53* mutation, and H1993 with a *p53* mutation and *c-MET* amplification; unpublished data). These findings suggest that FOSL1 promotes cancer cell proliferation and survival in a contextual manner (i.e., based on genetic and/or epigenetic status). Consistent with this notion, it was reported that the bromodomain and extra-terminal domain (BET) protein BRD4 promotes the proliferation and survival of certain HLAC cells (A549 and H23) by upregulating *FOSL1* (21). *FOSL1* knockdown phenocopied the inhibitory effect of BET inhibition on the growth of HLAC cells with KRAS mutations. However, depletion of high-level *FOSL1* expression in BET inhibition-insensitive *EGFR* mutant HLAC cells (e.g., H3255 and H1650) had no effect. Likewise, we found that, despite the elevated levels of FOSL1 in H1993 and CL1-5 cells, siRNA-mediated depletion of this transcription factor had no inhibitory effect on their growth and survival (data not shown). Together, these results suggest that HLAC cells with mutant KRAS are largely dependent on FOSL1 for their survival and maintenance. The observation that cells that did not bear KRAS mutations also expressed elevated levels of *FOSL1* expression, but its knockdown did not affect their growth and survival, indicates that FOSL1 may not be required to mediate

cancer cell growth and survival functions in the setting of non-KRAS mutations. FOSL1 largely dimerizes with JUN, which can form complexes with other FOSL1 counterparts, such as FOS, FOSB, and FOSL2 (FRA-2). Thus, it is possible that other FOS family members may compensate FOSL1-deficiency in regulating non-KRAS-mutant HLAC cell growth. Further studies are needed to determine whether FOSL1 targeting is only applicable to HLAC cells with KRAS mutations, and whether a combined targeting of FOSL1 and its counterparts is required to mitigate lung cancer with KRAS and other genetic mutation(s). It is noteworthy that studies performed with cell cultures often do not mimic *in vivo* conditions due to the lack of circulating and infiltrated immune and inflammatory mediators, and multiple cell-cell interactions. Thus, the lack of effect of FOSL1 deficiency on non-KRAS-mutant human lung cancer cell growth remains to be verified *in vivo* using non-Kras-mutant experimental models of lung cancer.

In summary, our studies show the importance of *FOSL1* for mediating KRAS-induced lung tumorigenesis *in vivo*, and suggest that the elevated expression of this transcription factor in patients with lung adenocarcinomas may be a prognostic marker for lung cancer deaths. Mechanistically, our data link AREG expression and regulation of antiapoptotic and antioxidative gene expression with FOSL1-mediated HLAC cell proliferation and survival, largely in the context of oncogenic KRAS mutations. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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