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Overexpression of RNA-binding protein CELF1 prevents apoptosis and destabilizes pro-apoptotic mRNAs in oral cancer cells

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CELF1 RNA-binding protein, otherwise called CUGBP1, associates and coordinates the degradation of GU-rich element (GRE) containing mRNA's encoding factors important for cell growth, migration and apoptosis. Although many substrates of CELF1 have been identified, the biological significance of CELF1-mediated mRNA decay remains unclear. As the processes modulated by CELF1 are frequently disrupted in cancer, we investigated the expression and role of CELF1 in oral squamous cancer cells (OSCCs). We determined that CELF1 is reproducibly overexpressed in OSCC tissues and cell lines. Moreover, depletion of CELF1 reduced proliferation and increased apoptosis in OSCCs, but had negligible effect in non-transformed cells. We found that CELF1 associates directly with the 3'UTR of mRNAs encoding the pro-apoptotic factors BAD, BAX and JunD and mediates their rapid decay. Specifically, 3'UTR fragment analysis of *JunD* revealed that the GRE region is critical for binding with CELF1 and expression of JunD in oral cancer cells. In addition, silencing of CELF1 rendered BAD, BAX and JunD mRNAs stable and increased their protein expression in oral cancer cells. Taken together, these results support a critical role for CELF1 in modulating apoptosis and implicate this RNA-binding protein as a cancer marker and potential therapeutic target.

Introduction

Apoptosis is a tightly regulated physiological process activated in response to various signals, including DNA damage, hypoxia and growth factor withdrawal. The BCL-2 family of proteins plays an integral role in apoptosis by modulating release of the pro-apoptotic cytochrome C from the mitochondria. Release of cytochrome c, in turn, leads to caspase activation and cell death.[1] Importantly, BCL2 family members can have pro-apoptotic and anti-apoptotic effects. BCL2 itself inhibits apoptosis by binding to pro-apoptotic family members, including BAX and BAK, and preventing them from inducing cytochrome C release.[2] The BAD protein also has pro-apoptotic functions but acts by sequestering BCL2 and preventing it from interacting with BAX and BAK.[3] Therefore, the balance of BCL2 family members in the cell determines when and whether cells enter the apoptosis pathway. Moreover, deregulation of apoptotic genes has been linked to cancer progression. For example, overexpression of BAX, BAK and BAD proteins promotes apoptosis in a variety of cell systems.[1,2,4-6] Given the importance of tight control of the apoptosis pathway, understanding the mechanisms by which the cell controls expression of BCL2-like proteins such as BAX, BAD and BAK is paramount. Transcriptional control of these potent regulators has been studied extensively, however less is known regarding their post-transcriptional regulation. In addition, JunD is a versatile AP-1 transcription factor known to activate or repress diverse target genes involved in apoptosis, angiogenesis and cellular differentiation.[7,8] Although JunD-mediated transcriptional activation has been extensively studied, the role of post-transcriptional regulation of JunD mRNA in apoptosis remains elusive.

Post-transcriptional events influence gene expression at multiple levels from mRNA processing through export to translation and decay. These events are controlled through interaction of RNA-binding proteins (RBPs) and/or miRNAs with the pre-mRNA and later with the mature transcript. CELF1 is one such RNA-binding protein that acts in the nucleus and cytoplasm to regulate alternative splicing, deadenylation, mRNA stability and translation.[9] Although CELF1 was first discovered as an RBP that is overexpressed and contributes to pathogenesis in myotonic dystrophy, several recent studies have implicated a role for CELF1 in cancer and apoptosis.[10-13] Deletion of CELF1 resulted in decreased cell viability, growth retardation, infertility and

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apoptosis in mice.[12] In addition, a CELF1 gene translocation has been linked with leukemogenesis.[14] In HeLa cells, knockdown of CELF1 induced caspase-3 activation.[15] Conversely, overexpression of CELF1 in HeLa cells prevented apoptosis under stress conditions by inducing expression of p21.[11] Moreover, overexpression of CELF1 in esophageal epithelial cells increased resistance to apoptosis and silencing CELF1 resulted in chemotherapy-induced apoptosis.[10] Lastly, CELF1 directly associates with several mRNAs that encode proteins involved in cell growth, cell cycle and apoptosis, including JunD.[15,16] However, to date, the extent to which CELF1 modulates apoptosis and how this might influence cancer progression remains unknown.

Here, we characterized the ability of CELF1 to regulate cell proliferation and apoptosis through interaction with mRNAs encoding pro-apoptotic factors such as *BAX*, *BAD* and *JunD*. We observed an overexpression of CELF1 in human oral squamous cell carcinomas (OSCCs). Also, knockdown of CELF1 in oral cancer cells reduced cell proliferation and enhanced entry into apoptosis. Surprisingly, knockdown of CELF1 in normal cells did not influence either the rate of cell proliferation or apoptosis. In OSCCs, CELF1 KD elevated translation and stability of mRNAs encoding pro-apoptotic factors. The association of CELF1 with the 3'UTRs of pro-apoptotic mRNAs was confirmed by RNA immunoprecipitation. These observations suggest that overexpression of CELF1 in tumors reduces the expression of pro-apoptotic genes that are essential for cell death, thereby affecting the balance between proliferation and apoptosis.

Results

Elevated expression of CELF1 in OSCC. To examine CELF1 expression in human oral cancer, we screened oral cancer tissue arrays that represented the most common benign, malignant and metastatic tumors originating from various anatomic sites within the oral cavity (**Table S1**). Using immunocytochemistry, we observed elevated CELF1 expression in a representative tongue OSCC tissue sample compared with normal adjacent tongue tissue (**Fig. 1A**). Expression of CELF1 increased ($p < 0.05$) as the tumor progressed from stage 1 to stage 4 (**Fig. 1B**; **Fig. S1**). Cancers showed overexpression of CELF1 in the proportional odds model ($p = 1.3 \times 10^{-5}$). The effect size, measured as log cumulative odds ratio comparing cancer vs. normal, was estimated to be 2.65 with a 95% confidence interval of (1.32, 2.56), determined as described previously.[17] Next, we measured *CELF1* mRNA and protein expression in human oral cancers, normal adjacent tissues and in various oral cancer cell lines (UM74A, UM74B, UM22A, UM22B, OSCC3 and OSCC15) using quantitative real-time PCR (qRT-PCR) and western blot analyses. Consistent with our OSCC tissue array analyses, *CELF1* mRNA in 13 tissue samples (**Fig. 1C**) and protein levels in five tissue sets (**Fig. 1D**) was overexpressed in all oral cancer tissues compared with adjacent normal tissues. We also observed a significant ($p < 0.05$, $p < 0.01$) increase in *CELF1* mRNA (**Fig. 1E**) and protein levels (**Fig. 1F**) in oral cancer cells compared with normal human oral keratinocytes (HOK). These results suggest that in OSCC, expression of CELF1 is elevated compared with normal cells.

CELF1 depletion induces apoptosis in tumor cells, but not in normal cells. Recent studies have characterized CELF1-associated mRNAs in various cell types.[15,18] These studies revealed that many of the mRNAs associated with CELF1 encode factors involved in cell proliferation and apoptosis pathways, with a subset of these involved in pro-apoptotic functions. We hypothesized that CELF1 exerts a role in apoptosis through post-transcriptional regulation of pro-apoptotic mRNAs. First, to test whether CELF1 is required for cell proliferation, we measured cell proliferation by MTT [3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay following CELF1 siRNA knockdown. Knockdown of CELF1 in oral cancer UM74B cells (**Fig. S2A**) reduced cell proliferation more than 5-fold after 72 h (**Fig. 2A**) compared with controls transfected with a scrambled siRNA. Moreover, silencing CELF1 in two additional oral cancer cell lines, UM11B and UM22A, reduced their rate of cell proliferation (**Fig. S2B**). These results indicate that CELF1 is necessary for cell proliferation in oral cancer cells. Surprisingly, knockdown of CELF1 in normal immortalized oral keratinocytes (OKF6tert1) (**Fig. S2C**) did not alter the rate of cell proliferation by MTT assay (**Fig. 2B**). In addition, siRNA-silencing of CELF1 (**Fig. S2D**) in other non-cancerous HaCaT cells (immortalized human keratinocytes) exhibited no change in cell growth (**Fig. S2E**). Next, to determine whether CELF1-mediated reduction in cell proliferation influenced apoptosis in normal HOK and oral cancer UM74B cells, we measured apoptosis following knockdown of CELF1 by staining with an enhanced green fluorescent protein (EGFP) fusion of annexin V. Knockdown of CELF1 did not alter the apoptosis rate of normal immortalized oral keratinocytes; (**Fig. 2C**) however, UM74B oral cancer cells (**Fig. 2D**) exhibited an approximate 9-fold increase in apoptosis following CELF1 knockdown (**Fig. 2E**, $p < 0.01$, bottom panel). Interestingly, CELF1 depletion induced cleavage of caspase 3, caspase 7 and poly (ADP-ribose) polymerase (PARP) in UM74B (**Fig. 2F**; **Fig. S2F**), UM11A and UM22B cells (**Fig. S2G**), but not in normal cells (**Fig. 2G**). The differing response of normal keratinocyte cells and cancer cells to CELF1 depletion is intriguing and suggests that CELF1 overexpression in cancer is important for proliferation and has anti-apoptotic influence.

CELF1 destabilizes *BAX, BAD* and *JunD* mRNAs in oral cancer cells. CELF1 is known to modulate mRNA decay rates for several of its target transcripts and generally acts as a destabilizing factor.[16] To ascertain whether suppression of CELF1 affects the decay of pro-apoptotic transcripts, we measured mRNA half-lives following inhibition of transcription with Actinomycin D. First, the qRT-PCR analysis showed that depletion of CELF1 significantly increased the abundance of *BAX*, *BAD* and *JunD* mRNA ($p < 0.05$) in comparison with control siRNA-treated cells (**Fig. 3A**). Second, knockdown of CELF1 elevated the half-lives of *BAX* (**Fig. 3B**), *BAD* (**Fig. 3C**) and *JunD* (**Fig. 3D**), but not GAPDH (**Fig. 3E**). Thus, CELF1 destabilizes *BAX*, *BAD* and *JunD* mRNA in oral cancer cells. Finally, to determine whether the *BAX*, *BAD* and *JunD* 3'UTRs were sufficient to confer CELF1-dependent transcript instability, we measured mRNA decay rates of the luciferase reporter mRNAs in UM74B cells. The 3'UTRs of *BAX*, *BAD* and *JunD* in control siRNA-treated cells degraded

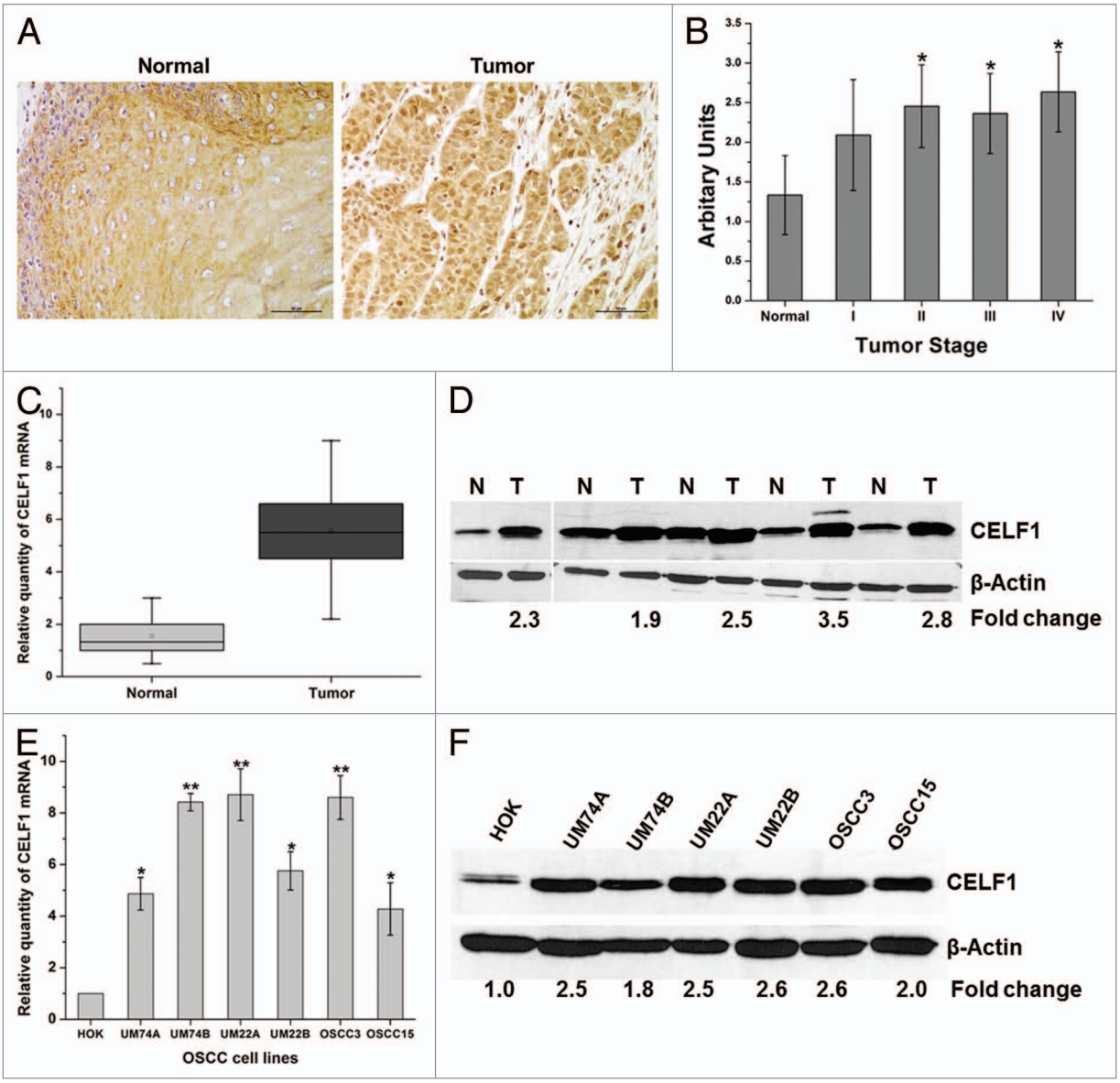

Figure 1. CELF1 is overexpressed in oral squamous cell carcinoma. (**A**) Determination of CELF1 expression using tissue microarray (TMA) samples of normal and HNSCC tissues. The tissue sections were subjected to immunohistochemistry using a primary monoclonal antibody to CELF1 followed by peroxidase-conjugated goat anti-mouse secondary antibody. Expression of CELF1 was relatively high in tumors (brown) and significantly over-expressed in the nucleus. Scale bar denotes 50 μm. (**B**) Measurements of total CELF1 accumulation in normal tissue samples compared with tumor stages I–IV. Stages I, II, III and IV (n = 13, 14, 21 and 18, respectively); normal (n = 9). (**C**) Relative *CELF1* mRNA levels in normal adjacent (n = 13) and tumor tissues (n = 13) normalized to β-actin. Box chart represents the relative expressions of CELF1 compared with normal adjacent tissue mRNA expression. (**D**) CELF1 protein expression in adjacent normal (N) and tumor (T) tissues. Signal intensity was quantified using densitometric analysis normalized to β-actin and is represented as fold change over normal adjacent tissues. The top band appears to be a contaminated band in one of the tumor samples. (**E**) Relative quantity of mRNAs measured with RT-qPCR. Total RNA was isolated from normal primary human oral keratinocytes (HOK) and HNSCC cell lines, (UM74A, 74B, 22A and 22B) OSCC3 and OSCC15. The cDNAs were synthesized and qPCR was performed. Data were normalized to β-actin and expressed as *CELF1* mRNA quantity relative to normal keratinocytes. (**F**) Immunoblot of CELF1 protein expression in HOK and HNSCC cell lines. Signal intensity was quantified by densitometric analysis and normalized to actin and expressed as fold change over the signal of normal keratinocytes (HOK). (*$p < 0.05$, **$p < 0.01$).

with half-lives of approximately 30, 60 and 30 min, respectively (**Fig. 3F**). Importantly, in CELF1-knockdown cells, the half-lives were significantly increased to 120, 110 and 130 min ($p < 0.05$) (**Fig. 3F**; **Fig. S3**) for the *BAX*, *BAD* and *JunD* 3'UTR reporters, respectively, while no significant change in the half-life of the GAPDH 3'UTR reporter was observed. To note, the half-life of luciferase-tagged 3'UTRs of *BAX*, *BAD* and *JunD* exhibit different time periods in comparison with full-length counterparts of these transcripts. We assume that this discrepancy might be due to the number of GRE motif's present in full-length transcripts of *BAX*, *BAD* and *JunD* in comparison with UTR's. Currently, we are working to resolve this issue. Thus, association of CELF1

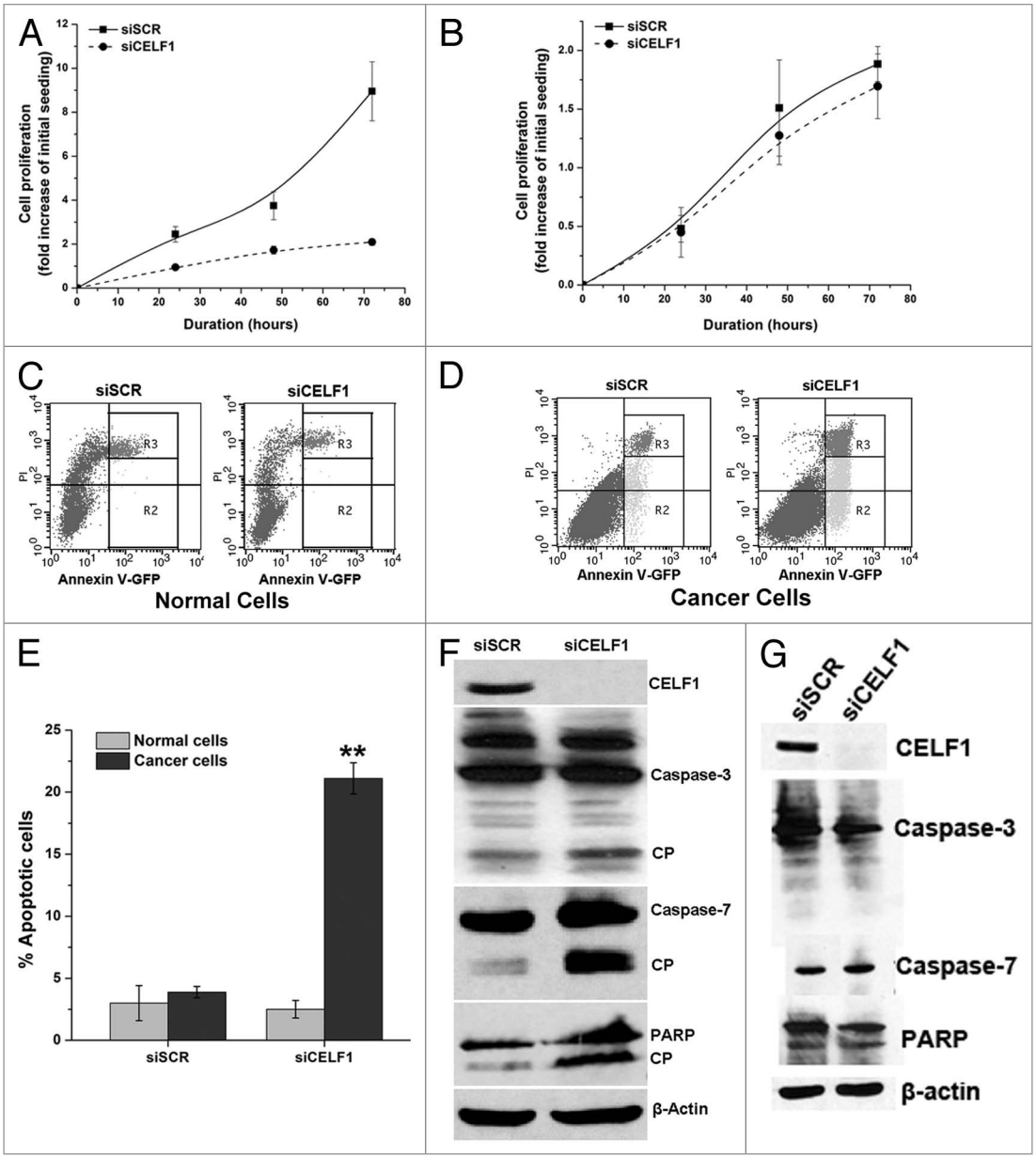

Figure 2. CELF1 depletion reduced proliferation and increases apoptosis in OSCCs, but not in normal immortalized cells. (**A**) Knockdown of CELF1 inhibits cell proliferation in the oral cancer cell line UM74B. The percentage of proliferating UM74B cells was quantified 24–72 h post-transfection of CELF1 siRNAs using the MTT cell proliferation assay (n = 3). (**B**) Knockdown of CELF1 did not change the rate of cell proliferation in the OKF6tert1 cells. The percentage of proliferating cells was quantified 24–72 h post-transfection of CELF1 siRNAs using the MTT assay. (**C**) OKF6tert1 cells were stained with annexin V-FITC and PI 48 h post-transfection and counted using flow cytometry. The number of apoptotic cells were determined for siCELF1- or siSCR-transfected cells. Values were normalized to untransfected cells. (**D**) UM74B cells were transfected with siCELF1 or siSCR and apoptosis was measured as described above. (**E**) Quantification of flow cytometry (**C and D**) data showing the percentage of apoptotic cells after transfection with siSCR or siCELF1 siRNAs. Values represent the mean ± SD from three independent experiments. (*$p < 0.05$). (**F and G**) Knockdown of CELF1 activates caspases. UM74B (**F**) and OKF6tert1 (**G**) cells were transfected with siRNAs (siSCR and siCELF1), followed by western blotting for caspases using anti-active caspase-3, -7 and poly (ADP-ribose) polymerase (PARP). β-actin served as a loading control. CP, cleaved product.

with the 3'UTRs of pro-apoptotic mRNAs enhances mRNA decay in oral cancer cells.

CELF1 associates with the 3'UTR of *BAX*, *BAD* and *JunD* in OSCCs. We hypothesized that CELF1 exerts its role in apoptosis through regulation of mRNAs encoding pro-apoptotic factors such as *JunD*.[15] We performed RNA IP in oral cancer cells using a monoclonal antibody against CELF1 and analyzed CELF1-interacting mRNA by qRT-PCR. We observed a significant enrichment of CELF1-bound *BAX*, *BAD* and *JunD* mRNA ($p < 0.05$), compared with IgG control beads (**Fig. 4A**). Interestingly, we did not find significant binding between CELF1 and its target mRNAs in normal OKF6tert1 cells (**Fig. S4**). In addition, we examined the association of luciferase reporter mRNAs bearing the 3'UTR of *BAX*, *BAD*, *JunD* or *GAPDH*

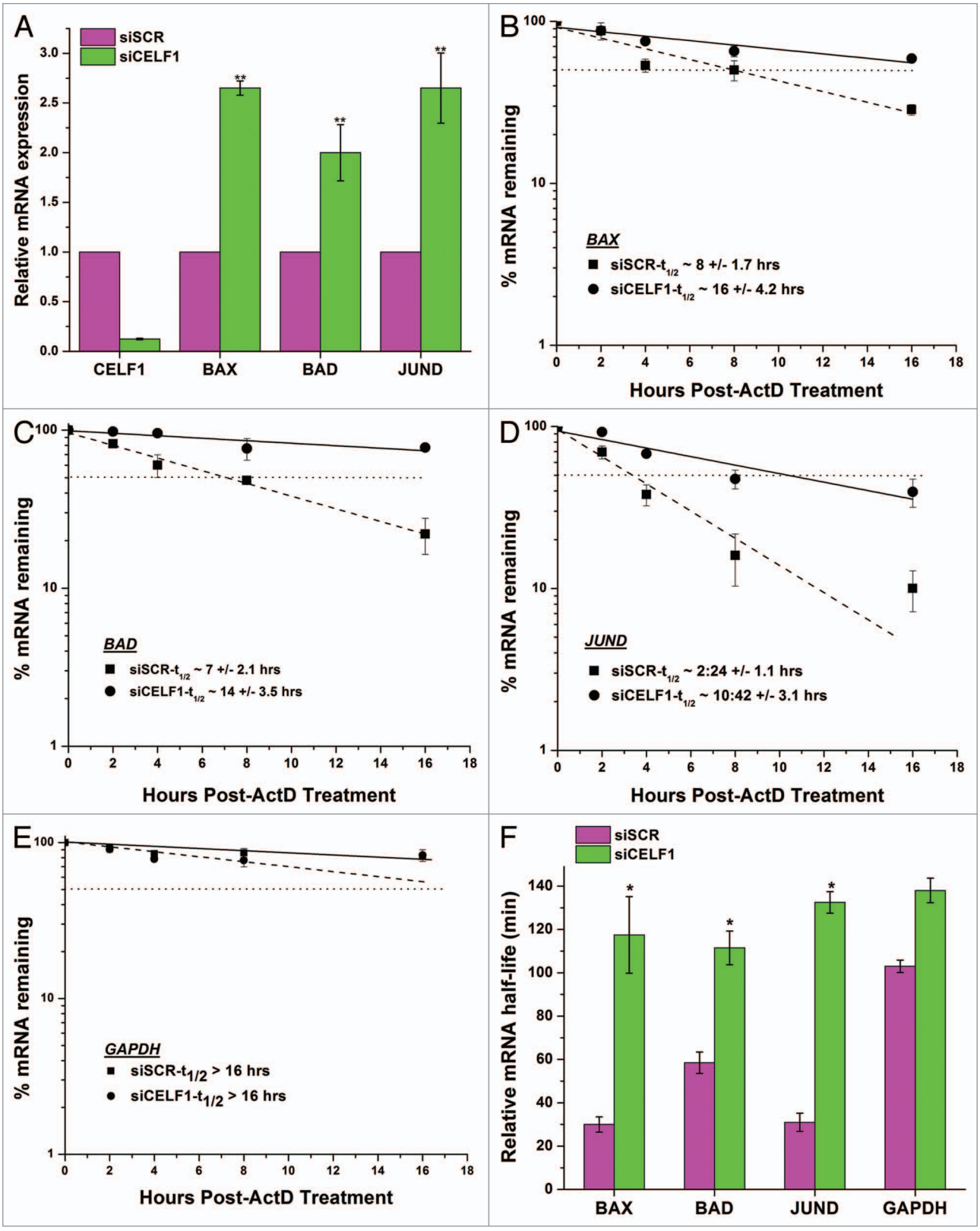

Figure 3. CELF1 is required for rapid decay of *BAX*, *BAD* and *JunD* transcripts in OSCCs. (**A**) The relative amounts of CELF1 and a panel of GRE-containing mRNAs expressed in UM74B cells transfected with CELF1 siRNA or control siRNA was measured with RT-qPCR. GAPDH was used as an endogenous control. (**B–E**) The decay rates of *BAX*, *BAD*, *JunD* and GAPDH (negative control) mRNAs in UM74B cells transfected with either siScr or siCELF1 using RT-qPCR following transcription inhibition with actinomycin D. Error bars denote the standard deviation of the mean of three independent experiments. (**F**) The half-lives of *BAX*, *BAD* and *JunD* UTR reporters were calculated based on the values obtained from RT-qPCR following transcription inhibition with actinomycin D. GAPDH serves as an control. Error bars denote the standard deviation of the mean of three independent experiments.

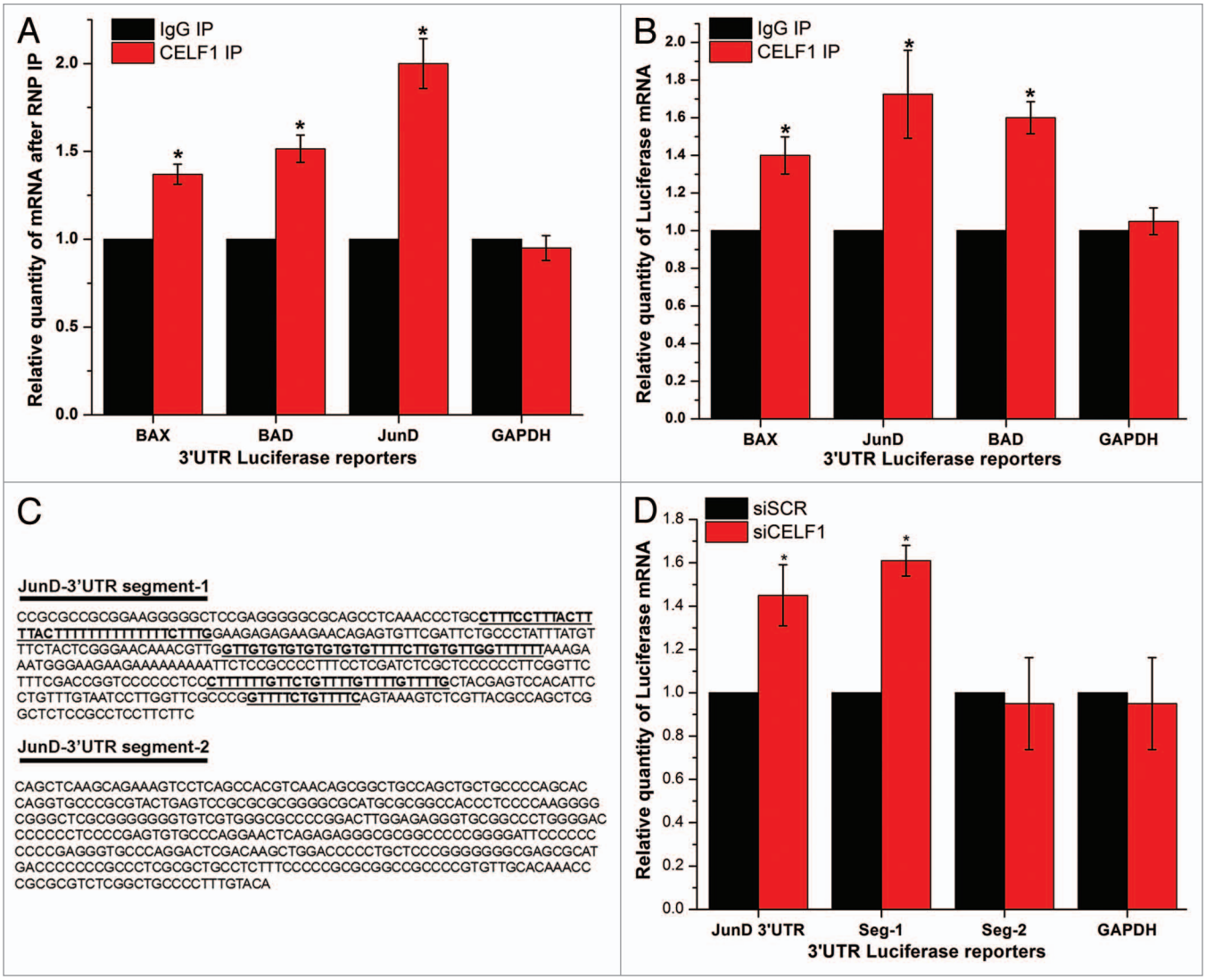

Figure 4. CELF1 associates with the 3'UTR of mRNAs encoding pro-apoptotic factors in OSCCs. (**A**) RNP IP of UM74B cells followed by RT-qPCR analysis was used to measure the relative quantity of *BAX*, *BAD* and *JunD* mRNAs. (**B**) Association of CELF1 with the 3'UTR of *BAX*, *BAD* and *JunD* mRNAs. RNP IP was performed 48 h post-transfection of UM74B cells with *BAX*, *BAD* and *JunD* 3'UTR fused to a luciferase reporter constructs. Luciferase mRNA was detected using RT-qPCR. The luciferase gene fused to *GAPDH* 3'UTR was used as a transfection and RT-qPCR control. (**C**) Schematic depiction of the 3'UTR of *JunD* segments used for CELF1 binding site identification. Segment-1 contains GRE motifs (underlined) and segment-2 does not contain GRE motifs. (**D**) Forty-eight hours after transfection of UM74B cells with luciferase-fused *GAPDH* 3'UTR plasmid or a full-length *JunD* 3'UTR, *JunD* segment-1 (seg-1) or seg-2, the levels of luciferase mRNA were measured after RNP IP using CELF1 antibody or control IgG beads. Luciferase-fused *GAPDH* 3'UTR served as a transfection and loading control. Values are the means ± SD from two independent experiments. (*$p < 0.05$).

with CELF1. UM74B cells were transiently transfected with each of the 3'UTR luciferase reporter constructs and extracts were prepared after 48 h. The extracts were subjected to RNA-IP with CELF1 antibody followed by qRT-PCR analysis. The mRNAs bearing the *BAX*, *BAD* and *JunD* 3'UTRs were significantly associated with CELF1, but the control mRNA bearing the GAPDH 3'UTR was not (**Fig. 4B**). To determine if CELF1 preferentially binds to the GU-rich region of the *JunD* 3'UTR, we created two luciferase reporters with fragments of *JunD* 3'UTR region and performed RNA IP with a CELF1 monoclonal antibody. Segment-1 (seg-1) contained consensus sequences of the GU-rich element "UGUUUGUUGU," and interacted with CELF1, whereas segment-2 (seg-2), which lacks GU-rich elements (**Fig. 4C**), did not appreciably interact with CELF1 (**Fig. 4D**). Together, these findings demonstrate that CELF1 associates with the GRE motif within the 3'UTRs of pro-apoptotic mRNAs.

Suppression of CELF1 enhances expression of BAX, BAD and JunD. We next determined the effect of CELF1 on expression of BAX, BAD and JunD proteins. Western blot revealed that the abundance of the corresponding proteins was also significantly higher in CELF1-depleted cells compared with controls (**Fig. 5A** and right panel depicts quantitative values). This increase in mRNA and protein abundance is likely mediated through the 3'UTR as all three luciferase reporters of *BAX*, *BAD* and *JunD* were associated with *CELF1*. To ascertain whether the expressions of these proteins are controlled at the translational level, we measured the luciferase

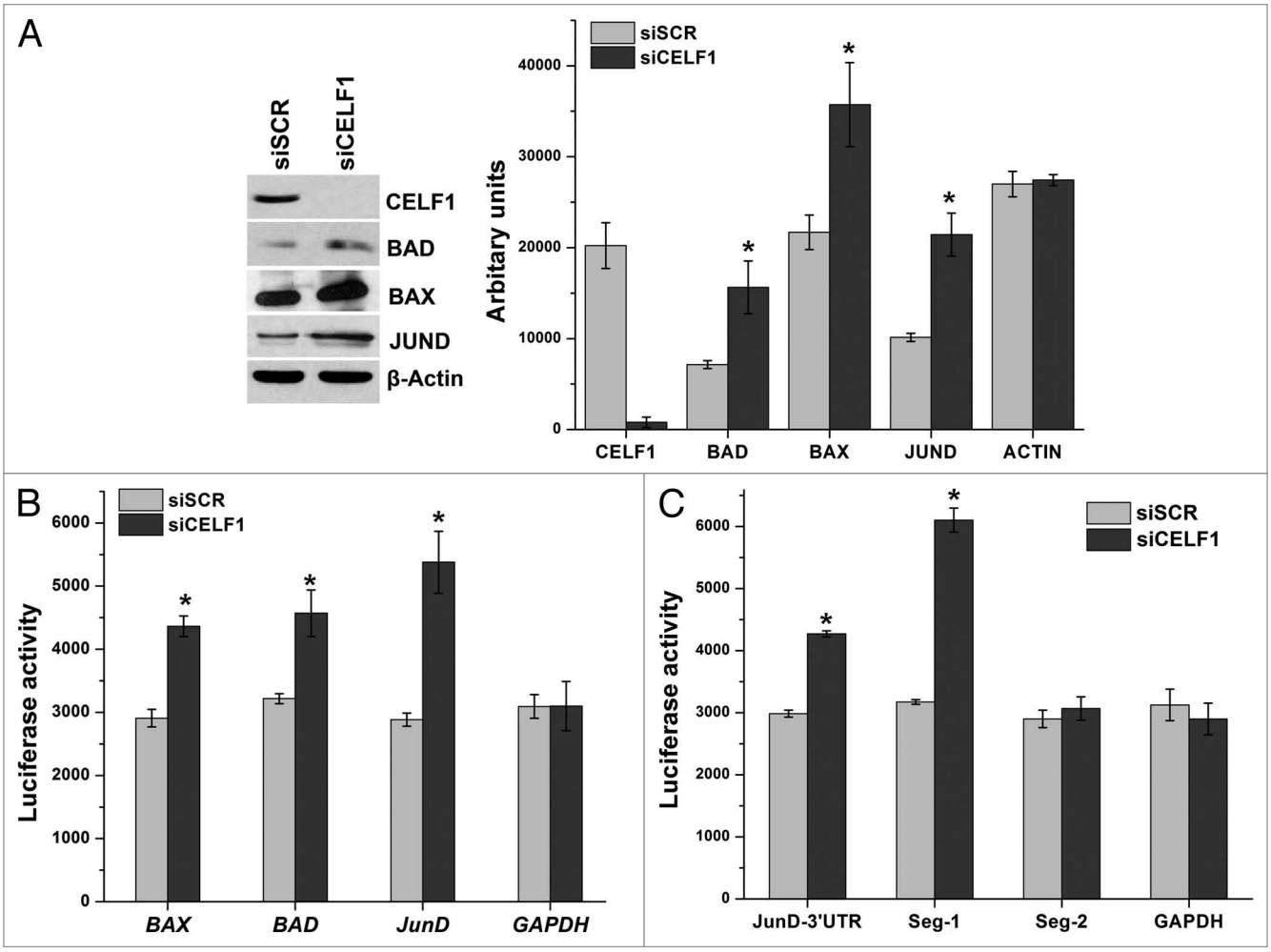

Figure 5. CELF1 depletion increases expression of *BAX*, *BAD* and *JunD*. (**A**) Forty-eight hours after transfection of UM74B cells with control siSCR or siCELF1, protein lysates were prepared to assess the levels of CELF1, *BAX*, *BAD* and *JunD* protein expression. β-actin served as a loading control. (Right panel) Graph depicts the quantitative value of the western blot data in triplicate analysis. (**B**) Forty-eight hours after transfection of UM74B cells with siSCR or siCELF1, cells were transfected with 3'UTRs as indicated, and after 48 h, the lysates were analyzed for luciferase activity using luminometer. (**C**) Forty-eight hours after transfection of UM74B cells with siSCR or siCELF1, cells were again transfected with 3'UTRs as indicated, and after 48 h, the lysates were analyzed for luciferase activity using luminometer. Values are the means ± SD from three independent experiments. (*$p < 0.05$).

activity of all three 3'UTR luciferase reporters of *BAX*, *BAD* and *JunD* in the absence of CELF1. As expected, we have observed a marked increase in luciferase activity of *BAX*, *BAD* and *JunD* compared with CELF1-knockdown cells (**Fig. 5B**) ($p < 0.05$). In addition, CELF1-knockdown cells significantly enhanced ($p < 0.05$) the luciferase activity of the reporter chimeric plasmid expressing GU-rich (*JunD*-FL and seg-1), but not the non-GU-rich fragment (seg-2) (**Fig. 3D**). Interestingly, CELF1 knockdown did not appreciably enhance the expression of *BAX*, *BAD* and *JunD* expression in normal OKF6tert1 cells (**Fig. S5**). Thus, CELF1 knockdown enhances the expression of JunD protein possibly through translational regulation in oral cancer cells.

Discussion

In UM74B cancer cells, CELF1 is overexpressed and prevents accumulation of pro-apoptotic transcripts by binding their 3'UTRs and inducing their decay. CELF1 knockdown resulted in an inappropriate stabilization of pro-apoptotic mRNAs, including *BAX*, *BAD* and *JunD* and induced apoptosis in oral cancer cells. Thus, overexpression of CELF1 in OSCCs prevents apoptosis at least in part by destabilizing pro-apoptotic mRNAs and this pathway appears to be essential for continued proliferation of these tumor cells. It seems likely that this role of CELF1 is cancer cell-specific since depletion of CELF1 in non-cancer cells has no appreciable effect on apoptosis (**Fig. 2C and G**) or proliferation (**Fig. 2B**).

Overexpression of RBPs in cancer. Overexpression of CELF1 is observed in a variety of oral cancer cells and tumor tissues (**Fig. 1**), suggesting that it could be a valuable prognostic or diagnostic marker. Similar observations have been made for other RBPs. For example, the HuR protein influences decay or translation of numerous mRNA targets involved in cell proliferation, survival, evasion of immune recognition, metastasis

and angiogenesis.[19] Like HuR, CELF1 and its target mRNAs are involved in post-transcriptional regulatory networks that control cell growth, activation and differentiation.[20] Because of this, inappropriate expression of a single RBP can impact a wide-range of cellular functions in a coordinated fashion to profoundly change the phenotype of the cell. In this case, we believe overexpression of CELF1 in cancer cells coordinates downregulation of pro-apoptotic pathways to facilitate continued cell proliferation.

CELF1 is also overexpressed in myotonic dystrophy and a number of other neuromuscular diseases.[9] However, our data suggest that the mechanism for CELF1 overexpression in OSCCs differs from that in myotonic dystrophy (DM) because we observed elevated CELF1 mRNA in OSCCs. In DM, CELF1 overexpression is mediated through hyperphosphorylation[21] and stabilization of the CELF1 protein with minimal, if any, effect on mRNA abundance. It will be interesting in the future to determine whether the increase in CELF1 mRNA occurs at the transcriptional or post-transcriptional level and to discover the factors behind this increase. One candidate would be miR-23a/b, which has been shown to target and downregulate CELF1 mRNA abundance during development,[22] and has been linked with cancer progression.[23] In addition, it has been shown that repression of CELF1 by miR-503 increased the sensitivity of intestinal epithelial cells to apoptosis.[24] These findings identify miR-503 as a regulator of CELF1 expression and a modulator of intestinal epithelial homeostasis.

Association of GRE mRNAs with CELF1 is critical for their expression in cancer cells. We have demonstrated that CELF1 associates with *BAX*, *BAD* and *JunD* transcripts and destabilizes them, thereby restricting expression of these pro-apoptotic proteins. This observation clearly indicates that CELF1 is an mRNA destabilzation factor in oral cancer cells. We cannot rule out CELF1 association with other mRNAs that codes for cell proliferation, for example, CELF1 association with several cancer processes including proliferation has been described very well by using gene ontology.[15] Interestingly, under certain stress conditions, CELF1 exhibited reduced association with its target mRNAs suggesting that CELF1 may be post-translationally modified in response to stress. For example, studies have shown that phosphorylation of CELF1 increases CELF1 protein stability and/or alters CELF1 association with its mRNA targets.[18,21,25,26] Thus, differential phosphorylation of CELF1 in cancer cells may control the fate of its target transcripts. In addition, other RBPs may have combinatorial RNA binding activity with these mRNA transcripts and regulate its stability. For example, it has been shown that polyamines modulate the stability of *JunD* mRNA in intestinal epithelial cells through binding with RNA-binding proteins, such as HuR and AUF1.[27] Interestingly, it was shown that both HuR and CELF1 share mRNAs targets.[16] *BAX* is a known target of HuR under stress,[28] suggesting that during stress, BAX may dissociate from CELF1 and associate with HuR. It is well-known that HuR undergoes phosphorylation during stress and mediates mRNA stability;[29] therefore, HuR could compete with CELF1 and associate with these targets and contribute to their mRNA stability in cancer cells. However, more specific cellular assays are required to support this hypothesis. Taken together, our study suggests that CELF1 facilitates targeting of pro-apoptotic mRNAs and, thereby, reduces apoptosis in tumor cells, suggesting that CELF1 acts as an apoptotic regulator.

Materials and Methods

Human tumor samples. Frozen oral cancer tissue with adjacent normal tissue samples were obtained from patients surgically treated in the Department of Otolaryngology-Head and Neck Surgery at the Medical University of South Carolina (MUSC) or purchased from US Biomax Inc. Samples were subjected to protein and RNA extraction for immunoblotting and qPCR analyses, respectively. The study was approved by the ethics committee of the MUSC Institutional Review Board, and samples were obtained after the appropriate informed written consent of the patient. Patients' identities associated with all tumor samples were removed prior to analyses. Frozen tumor tissues were microdissected to assure that > 80% of tumors contained OSCC.

Immunohistochemistry of oral tumor samples. Multiple cancer tissue (tumor microarray) slides along with different stages of OSCC tissue array slides were used. Immunohistochemistry was performed using a peroxidase technique after high-temperature antigen retrieval in citrate buffer. Primary CELF1 monoclonal mouse antibody was used at 1:200 dilutions (Millipore). Slides were incubated in secondary antibody (HRP-IgG mouse) for 30–45 min and washed with PBS, followed by counterstaining with hematoxylin. Mouse IgG antibodies for matched normal and tumor tissue samples were used as a negative control. All tissue array slides were analyzed independently by Dr Nisha D'Silva (author) according to the intensity of staining.

Cell lines, constructs, transfection experiments and luciferase assays. The human oral cancer cell lines (authenticated and genotyped, ref. 30) were maintained in Dulbecco's modified Eagle medium (DMEM-Hyclone) containing 10% fetal bovine serum (FBS) with 100 U/ml penicillin and 100 μg/ml streptomycin. Normal human oral keratinocyte cells (HOKs) (ScienCell) were grown in keratinocyte serum-free medium supplemented with BPE and EGF (Gibco, BRL). Immortalized human oral keratinocytes (OKF6tert1) were cultured and harvested under log phase conditions as described previously.[31] Human HaCaT cells were kindly provided by Dr Keith Kirkwood, Craniofacial Biology, Medical University of South Carolina. Cell cultures were maintained in humidified atmosphere with 5% CO_2 at 37°C. For CELF1-knockdown analysis, cells were transfected with siRNAs (20 nM) in medium containing 5% FBS. Then, either the control siRNA (20 nM; GTTCAATTGTCTACAGCTA) or siRNA-targeting CELF1 (20 nM; GAGCCAACCUGUUCAUCUA) (Dharmacon RNAi Technologies) was used. The siRNA transfections used the HiPerfect (QIAGEN) transfection reagent, following the manufacturer's protocol. Human 3'UTRs of *BAX*, *BAD*, *JunD* and its segments 1 and 2 and *GAPDH* were systematically identified and cloned into an optimized luciferase reporter vector system (www.switchgeargenomics.com/products/utr-reporter-collection/). The luciferase plasmids shown in

Figure 4 were subcloned again into NheI and XhoI sites in the pLightswitch plasmid (Switchgear Genomics) to express chimeric mRNAs spanning two of the luciferase *JunD* 3'UTR segments. These two segments were construced from *JunD* 3'UTR mRNA sequences. The luciferase *GAPDH* 3'UTR negative control was included in all assays. Each construct was transfected in triplicate separately with either CELF1 siRNA or the scrambled siRNA control. Plates were incubated at 37°C for 24 h post-transfection before being removed. One hundred microliters of Steady Glo luciferase assay reagent (Promega) were added to each well, and plates were incubated at room temperature for 30 min. Luminescence was measured using a LmaxII-384 Luminometer (Molecular Devices).

Proliferation and apoptosis assays. Cell proliferation rate was determined using MTT Cell Proliferation Assays (Invitrigen). Cell apoptosis assays were performed according to the manufacturer's protocol (annexin V-EGFP apoptosis detection kit, Biovision). Cells were trypsinized and centrifuged at 300 × *g* for 5 min and resuspended in 500 μl of 1 × binding buffer. Annexin V-EGFP (5 μl) and propidium iodide (PI, 5 μl; 0.05 mg/ml) were added to the suspension, incubated for 5 min in the dark and filtered through a nylon mesh to remove cell clusters. Fluorescence was measured using a FACS Calibar Flow Cytometer (Becton-Dickinson).

Immunofluorescence and western blot analysis. For immunocytochemical staining, cells grown on glass coverslips were fixed in 1% paraformaldehyde for 10 min. After washing three times for 5 min in PBS, cells were permeabilized with 0.05% Triton X-100 in PBS for 5 min and washed with PBS (three times for 5 min). After blocking with 10% normal donkey serum in PBS for 1 h at room temperature, the coverslips were treated with the specified primary antibodies (CELF1 and caspase-3) at 1:100 dilution overnight at 4°C. Subsequently, cells were incubated with dye-conjugated (Alexa 568, Alexa Fluor 647) secondary antibodies and labeled phalloidin for 2 h. After washing (five times for 5 min) in PBS, the coverslips were mounted with DAPI mounting medium (Invitrogen). Cells were imaged using an Olympus 1X81 confocal microscope.

For western blotting, protein extracts were resolved with SDS/PAGE. Cells were lysed by vortexing 4–5 times in RIPA buffer [2 mM TRIS-HCl, 30 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, complete protease inhibitor cocktail (Roche), 1% NP-40, pH 7.6] at 10 min intervals, each at 4°C, followed by centrifugation at 12,000 × *g* for 30 min at 4°C. Supernatants were mixed with an equal volume of 2 × Laemmli's buffer and heated for 5 min at 95°C. Total protein was estimated using a Bradford assay.[32] Twenty to 60 μg of protein were resolved on 10–12% SDS-PAGE gels and transferred onto PVDF membranes. Blots were pre-incubated with PBS containing 5% skim milk prior to incubation with primary antibody against the target protein for 1–3 h at room temperature or overnight at 4°C. After incubation, the blot was washed four times with PBS containing 0.1% Tween 20 (PBS-T) and incubated with PBS-T containing 1:10,000 diluted HRP-conjugated secondary antibodies for 1 h at room temperature. After additional washing with PBS-T, immune complexes were visualized using the ECL system (Pierce). Blots were stripped and re-probed with anti-β-actin antibody as described above. Western blot analyses were performed using antibodies specific to CELF1 (Millipore), PARP (Cell Signaling), caspase-3, -7 and -8 (Cell Signaling) and Jun-D (Abcam) as suggested by the manufacturer's protocol.

RNA extraction and qPCR. Total RNA was prepared from oral cancer tissues and HNSCC cell lines using the RNeasy mini kit (QIAGEN). qPCR for all mRNA targets was performed using an Applied Biosystems StepOne Plus system with the SYBR green master mix RT-PCR kit (SA Biosciences). Primer sequences are provided in **Table S2**.

CELF1 RNP IP analysis. CELF1 RNP IP was performed as previously described,[33] with some modifications. Briefly, cell lysates were prepared from exponentially growing UM74B cells. Equal amounts of protein were used (100–300 μg). CELF1 monoclonal antibody (Millipore) or isotype control IgG (Santa Cruz) were pre-coated onto protein A/G Sepharose beads (PAS) and extensively washed using NT2 buffer [50 mM Tris–HCl, 150 mM NaCl, 1 mM $MgCl_2$, 0.05% Nonidet P-40 (NP-40), pH 7.4]. Lysates were pre-absorbed with IgG (30 μg) and then removed by addition of PAS beads. Individual pull-down assays were performed at 4°C for 1–2 h to minimize potential reabsorbing of mRNAs. For RNA analysis, the beads were incubated with 1 ml NT2 buffer containing 20 U RNase-free DNase I (15 min, 30°C), washed twice with 1 ml NT2 buffer and further incubated in 1 ml NT2 buffer containing 0.1% SDS and 0.5 mg/ml proteinase K (15 min, 55°C) to digest the proteins bound to the beads. RNA was extracted using phenol and chloroform, and precipitated in the presence of glycogen. For analysis of individual mRNAs, the RNA isolated from the IP was subjected to reverse transcription (RT) using random hexamers and SuperScriptII reverse transcriptase (Biorad). Amplification and quantification of the PCR products were performed using the Applied Biosystems Stepone Plus system (Applied Biosystems) and SYBR Green PCR Master Mix (SA Biosciences). Input *GAPDH* mRNA was used as a loading control.

Statistical analysis. Data were expressed as the mean ± the standard deviation. Two-sample t-tests with equal variances were used to assess differences between means. Results with p values less than 0.05 or 0.01 were considered significant.

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Supplemental Material

Supplemental material may be downloaded here:
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