USF-1 regulates ILEI transcription

Interleukin-like EMT inducer (ILEI) promotes melanoma invasiveness and is transcriptionally up-regulated by upstream stimulatory factor-1 (USF-1)

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ABSTRACT

Interleukin-like EMT Inducer (ILEI, FAM3C) is a secreted factor that contributes to the epithelial-to-mesenchymal transition (EMT), a cell biological process that confers metastatic properties to a tumor cell. However, very little is known about how ILEI is regulated. Here we demonstrate that ILEI is an in vivo regulator of melanoma invasiveness and is transcriptionally up-regulated by the upstream stimulatory factor-1 (USF-1), an E-box-binding, basic-helix-loop-helix family transcription factor. shRNA-mediated knockdown of ILEI in melanoma cell lines attenuated lung colonization but not primary tumor formation. We also identified the mechanism underlying ILEI transcriptional regulation, which was through a direct interaction of USF-1 with the ILEI promoter. Of note, stimulation of endogenous USF-1 by UV-mediated activation increased ILEI expression, whereas shRNA-mediated USF-1 knockdown decreased ILEI gene transcription. Finally, we report that knocking down USF-1 decreases tumor cell migration. In summary, our work reveals that ILEI contributes to melanoma cell invasiveness in vivo without affecting primary tumor growth and is transcriptionally up-regulated by USF-1.

The three most commonly mutated genes in melanoma are BRAF, NRAS, and NF1, all components of the RAS-RAF-MEK-ERK signaling pathway (subsequently referred to as the MEK signaling pathway) (1). Accordingly, MEK signaling plays a major role in melanoma biology by regulating diverse processes such as pigmentation, apoptosis, and senescence (2-9). At a molecular level, MEK signaling affects many transcription factors including the basic helix-loop-helix-leucine zipper (bHLH LZip) transcription factor microphthalmia-associated transcription factor (MITF) (3,7). MITF binds to E-box motifs (CATGTG) and activates the transcription of the pigment producing gene PMEL, which encodes the premelanosome protein, and cell cycle genes such as CDK2, which encodes the cyclin-dependent kinase 2 (10,11). In addition to MITF, MEK signaling affects other bHLH LZip family transcription factors such as upstream stimulatory factor 1 (USF-1) (12,13). USF-1 binds to E-box motifs (CACGTG) and activates pigmentation genes in response to UV (14,15).

Interleukin-like EMT Inducer (ILEI, FAM3C) is a secreted cytokine-like molecule that contributes to the epithelial-to-mesenchymal transition (EMT) (16). EMT is a cell biological process in which epithelial cells with apical-basal polarity undergo cytoskeletal rearrangement to become motile...
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mesenchymal cells (17). This process is thought to contribute to chemoresistance and metastasis. While melanoma cells do not undergo a traditional EMT, they utilize a similar process known as phenotype switching. This is a process in which melanoma cells interconvert between a proliferative MITF-high state and an invasive MITF-low state (18-24). Recently, we have described a contribution of ILEI to the invasive MITF-low phenotype in vitro (25). Additionally, we showed that phenotype switching between the proliferative MITF-high and invasive MITF-low state modulates ILEI mRNA expression. The molecular regulation of ILEI has focused on post-transcriptional mechanism including translational regulation by hnRNP-E1/TGF-β and proteolytic processing by plasmin (26-30). However, not much is known about transcriptional regulation of ILEI.

Here, we report that ILEI contributes to melanoma cell line invasiveness in vivo and build on our previous finding that vemurafenib (BRAFi) decreases ILEI mRNA expression to characterize the molecular details of ILEI transcriptional regulation by USF-1 (25).

RESULTS AND DISCUSSION

ILEI regulates lung colonization but not primary tumor growth.

Previously we found that knockdown of ILEI contributes to melanoma invasiveness in vitro whereas ILEI had no effect on proliferation (25). Here we used the same ILEI modulated cell lines and conducted in vivo flank and tail vein injections to measure primary tumor formation or lung colonization, respectively. In the flank injection experiment we found no significant difference in primary tumor growth (Fig 1A-B), but we observed in the tail vein injection experiment that ILEI knockdown significantly attenuated lung colonization (Fig 1C). We did not observe a difference in the size of the lung colonies formed by either shSCR or shILEI cell lines suggesting that the ability to invade the lung was affected rather than the ability to grow metastatic colonies in the lung (Fig 1D). Based on these results we conclude that ILEI specifically regulates melanoma invasiveness in vivo without affecting primary tumor growth.

Vemurafenib inhibits ILEI expression.

To date there are three known mechanisms of ILEI regulation: the first is translational regulation of ILEI by TGF-β/AKT2/hnRNP-E1, the second is degradation of ILEI by the ubiquitin/proteasome system, and the third is an autophagy-mediated increase in ILEI protein expression (16,25,26,30-33). Nothing has been reported about the mechanistic basis of ILEI transcription. None of the previously established mechanism appears to be responsible for ILEI regulation by vemurafenib (25). Herein, we sought to determine the mechanism of ILEI transcriptional regulation.

First, we treated WM983B melanoma cell lines with vemurafenib, an inhibitor of V600E BRAF, and observed that ILEI protein and mRNA expression decreased at 24h (Fig 2A, B) (25). We also conducted RT-PCR using primers targeting an intronic sequence of ILEI to amplify ILEI pre-RNA (Fig 2B). If ILEI mRNA levels are regulated post-transcriptionally by miRNAs, we expect that ILEI pre-RNA should not be affected by vemurafenib. However, if ILEI mRNA levels are regulated transcriptionally, we expect that ILEI pre-RNA should go down upon vemurafenib treatment. We saw that vemurafenib treatment decreased both ILEI total RNA and pre-RNA, suggesting that vemurafenib affected ILEI transcription (Fig 2B). In order to confirm that these findings were not due to off-target effects of vemurafenib we conducted a control experiment in which we used the BRAF WT WM3918 melanoma cells. Vemurafenib is a specific inhibitor of mutant BRAF, and it does not affect WT BRAF (34). Therefore, we treated BRAF WT WM3918 melanoma cells with vemurafenib, which should retain all the non-specific effects of vemurafenib without the BRAF-specific effects. We found that vemurafenib did not affect ILEI protein expression or ERK phosphorylation in BRAF
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WT WM3918 melanoma cells (Fig 2C). We confirmed these findings by a quantitative method using real-time qPCR and found that vemurafenib decreased ILEI expression in *BRAF* mutant 501-Mel and 1205Lu but not in *BRAF* WT WM3918 cells (Fig 2D). Considering that ILEI is a secreted cytokine, we wanted to confirm the physiological relevance of vemurafenib-mediated ILEI inhibition by conducting immunoblot analysis of the conditioned medium. These results confirmed that vemurafenib decreased secreted ILEI levels (Fig 2E). Finally, in order to further determine if this effect is specific for the MEK pathway we used a second inhibitor of the MEK pathway (U0126, MEKi) and observed decreased ILEI expression by U0126 (Fig 2F). Based on these results we conclude that vemurafenib inhibits ILEI mRNA expression, and that this effect is dependent on the presence of oncogenic *BRAF* V600E mutation.

**Vemurafenib inhibits ILEI at the transcriptional level.**

We cloned the ILEI promoter from 2,300 base pairs upstream of the transcription start site (TSS) to 80 base pairs upstream of the TSS into pBV-Luc (Fig 3A) (35). We transfected 501-Mel cells with the ILEI promoter luciferase reporter construct along with a control renilla construct and treated the cells with vemurafenib (Fig 3B). We found that vemurafenib decreased the ILEI promoter activity in a dose-dependent manner. As further controls we tested the ILEI promoter construct with the MEK inhibitor U0126 and found that U0126 decreased promoter activity (Fig 3C). However, when we tested the ILEI promoter construct with the PI3K inhibitor LY-294002, we found that promoter activity was not affected (Fig 3D). Additionally, we cloned the ILEI 3’-UTR from the stop codon to the end of the mRNA at 1,620 base pairs into pmirGLO Dual Luciferase (Fig 3E). We transfected 501-Mel cells with the ILEI 3’-UTR construct and treated the cells with vemurafenib (Fig 3F). We found that vemurafenib did not affect the ILEI 3’-UTR construct. From these experiments we concluded that vemurafenib regulates ILEI mRNA at the transcriptional level.

Since vemurafenib regulates ILEI transcription, we hypothesized that a cis element in the ILEI promoter region regulates this phenomenon. We analyzed a 2,300 base pair sequence at the ILEI promoter from the TSS for various transcription factor motifs using the JASPAR database (36). Previously we had found that ILEI is highly expressed in MITF-low invasive melanoma cell lines, so we focused our transcription factor search on regulators of the MITF-low invasive state (E-box [ZEB1/2], JUN, and TEAD4) (20,21,23,37-39). We found 11 putative E-box, 5 JUN, and 3 TEAD4 sites in the ILEI promoter, and generated successive 5’-deletions of the ILEI promoter reporter (Fig 4A). We transfected 501-Mel cells with these constructs and observed that the truncation from -204 to -150 reduced the luciferase activity to empty promoter control levels. There was an E-box motif in this region, so we next wanted to know if this E-box was specifically important for ILEI promoter activity or if any E-box would suffice. Thus, we used our longest ILEI promoter reporter (-2300 to -80) and specifically mutated the E-box consensus site at -163 from CACGTG to CAAATG. Again, we observed a marked inhibition in luciferase activity (Fig 4B). From these experiments we concluded that an E-box 163 bp upstream of the ILEI TSS is critical to basal ILEI promoter activity.

Next, we wanted to know the role of this E-box motif in the vemurafenib-mediated regulation of ILEI expression. We transfected 501-Mel cells with the ILEI promoter truncation constructs and treated the cells with vemurafenib (Fig 5A). We found that truncation of base pairs -204 to -150, which includes an E-box motif, eliminated ILEI promoter vemurafenib responsiveness. The -204 to -150 region of the ILEI promoter contains 48 base pairs in addition to the E-box, so we mutated the E-box in the -300/-80 construct. We sequenced the construct and analyzed the sequence in the JASPAR database to find that our mutations abolished all putative binding sites in this region (data
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not shown). We conducted luciferase assays and observed that mutation of the E-box similarly eliminated vemurafenib responsiveness (Fig 5B). From these experiments we concluded that vemurafenib inhibits ILEI at the transcriptional level through an E-box 163 bp upstream of the ILEI TSS.

**USF-1 directly regulates ILEI transcription.**

Considering the importance of the E-box motif to ILEI expression, we wanted to identify the trans-acting factor regulating this effect. To this end, we overexpressed different transcription factors known to bind E-box motifs and found that USF-1 induced ILEI promoter activity, but not c-MYC, N-MYC, L-MYC, TFEB, TFE3, MITF, CREB3L2, ID2 (Fig 6A). USF-1 activates transcription through the E-box motif, but also through a pyrimidine-rich initiator sequence (Inr) (40). The ILEI promoter sequence has an Inr sequence 6 bp downstream of the E-box (Fig 6B). Thus, we generated Inr mutant ILEI promoter constructs in addition to our E-box mutants to see if USF-1 regulated ILEI promoter activity directly through the USF-1 binding sites (Fig 6C). We observed that USF-1 induced ILEI promoter activity in the WT promoter, the E-box mutant promoter, the initiator mutant promoter, but not the E-box and Inr double mutant promoter (Fig 6D). From these experiments we concluded that USF-1 directly regulates ILEI promoter activity.

We wanted to further prove a direct role of USF-1 in ILEI regulation so we pursued a potential interaction between the ILEI promoter and USF-1. We conducted streptavidin pulldown experiments using 5’-biotin tagged ILEI promoter constructs either wild-type or mutant for the E-box (200 to 110 base pairs upstream of ILEI TSS, Fig 7A) and 501-Mel melanoma cell nuclear extract. We found that USF-1 can be isolated from cell nuclear extract using the wild-type ILEI promoter, but not the E-box mutant ILEI promoter, or a control random 60 base pair oligonucleotide (SCR, Fig 7A).

Interestingly, the E-box mutation was sufficient to abolish binding by the streptavidin pulldown experiment, but the E-box mutation was not sufficient to abolish USF-1-mediated induction of the ILEI promoter reporter (Fig 6D). We speculate either that 1) USF-1 still binds the ILEI promoter at levels that are undetectable by our streptavidin pulldown assay, or 2) endogenous USF-1 binds only to the E-box but overexpressed exogenous USF-1 provides excess USF-1 to bind both the E-box and the Inr. This is supported by the finding that basal ILEI promoter activity is affected more by the mutation of the E-box than the Inr (Fig 6D). This is also consistent with previously published findings that show the E-box is the high-affinity USF-1 binding site, whereas the Inr is the low-affinity binding site (41).

In order to confirm that the interaction between USF-1 and the ILEI promoter was physiologically relevant, we conducted chromatin immunoprecipitation using USF-1 antibody and PCR primers flanking the ILEI promoter E-box (Fig 7B). We confirmed the efficacy of our assay by showing that USF-1 IP can detect a segment of the TYR promoter (15), and we detected an interaction between USF-1 and the ILEI promoter (Fig 7B). However, we found that a segment of the HO1 promoter, which is a USF-1 interaction reported in epithelial cells, was not detected in our melanoma cells (42). From these experiments we concluded that USF-1 directly interacts with the ILEI promoter and this interaction is E-box-dependent.

**USF-1 in vemurafenib-regulated ILEI expression.**

Thus far we have shown that vemurafenib inhibits ILEI transcription through an E-box motif, and that USF-1 directly regulates ILEI transcription through the same E-box motif. Next, we wanted to understand the role of USF-1 in vemurafenib-mediated regulation of ILEI transcription.

For this we employed the streptavidin pulldown assay using 5’-biotin tagged ILEI promoter constructs wild-type or E-box mutant in cells treated with vemurafenib. First,
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we validated the binding of USF-1 to the ILEI promoter (DMSO condition) and showed that vemurafenib treatment abolished USF-1 interaction with the ILEI promoter (Fig 8A). Importantly, we noticed by comparing the Input lanes that USF-1 expression is decreased by vemurafenib treatment. We used qPCR to confirm that vemurafenib represses the mRNA levels of USF-1 (Fig 8B).

If vemurafenib inhibits ILEI expression by repressing USF-1, overexpression of USF-1 should rescue vemurafenib-mediated inhibition of ILEI. Thus, we overexpressed USF-1, treated with vemurafenib, and tested ILEI promoter activity. We found vemurafenib-regulation of ILEI expression in vector overexpressing cells, but not in USF-1 overexpressing cells (Fig 8C). In fact, we observed that vemurafenib treatment actually induced ILEI promoter activity in the context of USF-1 overexpression. We propose the following explanation: in the absence of exogenous USF-1, vemurafenib decreases USF-1 expression to inhibit ILEI transcription (Fig 8B), but in the presence of exogenous USF-1, vemurafenib activates p38 kinase to activate USF-1 and counterintuitively activate ILEI transcription (15,20).

Finally, we confirmed these results with the endogenous ILEI by overexpressing USF-1, treating with vemurafenib, and conducting PCR analysis. While the rescue was not complete, we observed that USF-1 overexpression rescues vemurafenib inhibition of ILEI (Fig 8D). From these experiments we concluded that the mechanism of vemurafenib-mediated inhibition of ILEI is through downregulation of USF-1 mRNA.

Role of endogenous USF-1 in ILEI expression.

We wanted to assess if endogenous USF-1 had a role in ILEI expression. USF-1 is known to be regulated by stress-mediated p38 MAPK. Mechanistically, p38 phosphorylates USF-1 on threonine 153, which activates its transcriptional activity for target genes including TYR (15). Thus, we used UV treatment as a model of endogenous USF-1 activation and observed that UV increased ILEI mRNA both by RT- and qPCR (Fig 9A-B). Furthermore, we wanted to know if UV/USF-1/ILEI regulation was intact in non-melanoma cell lines. We used HMLE human mammary epithelial cells and observed that UV increased ILEI mRNA by qPCR (Fig 9C). In addition to activation of endogenous USF-1, we wanted to test the effect of inhibiting endogenous USF-1. We used two different shRNA molecules specific for USF1 and observed that knockdown of endogenous USF-1 inhibits ILEI expression (Fig 9D). From these experiments we concluded that endogenous USF-1 regulates ILEI expression.

Finally, we wanted to know if USF-1 regulation of ILEI had any biological significance. Given the importance of ILEI to the invasive melanoma phenotype (Fig 1), we tested the contribution of USF-1 to wound healing. We observed that USF-1 knockdown in 501-Mel melanoma cells attenuates wound healing (Fig 10A, B). From these experiments we concluded that USF-1 regulates migration in melanoma cell lines.

In summary, we have described the following novel findings: ILEI regulates melanoma invasiveness in vivo, vemurafenib inhibits ILEI at the transcriptional level through a specific E-box sequence, USF-1 directly interacts with this E-box in the ILEI promoter, that this interaction is abolished upon vemurafenib treatment, and finally that endogenous USF-1 contributes to ILEI expression and the invasive melanoma phenotype. Through these findings we have established a novel regulatory mechanism for ILEI expression by USF-1, and based on this previously unknown context for ILEI expression we speculate on the following new biological functions for USF-1 and ILEI (Fig 11).

While ILEI has been thoroughly described as a tumor autonomous regulator of EMT and invasion, the role of ILEI in paracrine signaling is still unclear (25,28,29,33,43-45). Our characterization of the novel vemurafenib-USF-1-ILEI regulatory axis has implications for paracrine ILEI signaling. Given the impact of vemurafenib on
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*BRADF* mutant melanoma, the mechanisms of vemurafenib-mediated tumor killing have been thoroughly characterized. For instance, mouse models have shown that vemurafenib induces apoptosis of tumor suppressive regulatory T cells while simultaneously promoting CD8 T cell-mediated killing of tumor cells (46). Other models have shown that vemurafenib increases the recruitment of macrophages to *BRADF* mutant melanomas (47). Combining our finding that vemurafenib inhibits USF-1/ILEI with the established finding that vemurafenib regulates the tumor microenvironment, we suggest that both USF-1 and ILEI could be regulators of the tumor immune microenvironment. Future studies should seek to elucidate the role of USF-1/ILEI in this paracrine signaling context.

Additionally, USF-1 is primarily known as a regulator of glucose and lipid metabolism and is poorly described as an oncogene (48-50). In melanoma USF-1 is known as a stress responsive transcription factor involved in UV-induced, but not MITF-mediated constitutive pigmentation (51). The non-overlapping function of USF-1 with MITF in pigmentation suggests that USF-1 could play other roles in melanoma biology when MITF is poorly expressed, such as in MITF-low invasive melanoma cells. These cells are characterized by the lack of MITF but not by the presence of any major transcription factor, although ZEB1 and JUN have been proposed (20,21,23,37,38). Considering that ILEI is a regulator of MITF-low cells we speculate that USF-1 could be a novel transcription factor regulating the MITF-low invasive state (Fig 11) (25). Future work should address the role of USF-1 in melanoma phenotype switching.

**EXPERIMENTAL PROCEDURES**

**Animal Studies**

All procedures were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina. Lung colonization experiments were conducted as follows: tail vein injection into 6-8 week old mice were performed with 1205Lu cells expressing shSCR or shILEI 3 (1 x 10^5 cells in 100 µL of phosphate-buffered saline). Cells were previously characterized (25). After 8 weeks, mice were sacrificed, and lungs were harvested. Organs were formalin-fixed and paraffin-embedded. Histopathological analysis was conducted by Hollings Cancer Center Biorepository & Tissue Analysis Shared Resource. Lung colonies were counted by two individuals.

**Constructs**

ILEI reporter constructs were cloned as follows: ILEI promoter sequence from 2,300 base pairs upstream of the predicted transcription start site to 80 base pairs downstream of the stop codon was amplified by PCR from 501-Mel genomic DNA with 5’ KpnI and 3’ NheI primers and ligated into pBV-Luc (pBV-Luc vector (a gift from Bert Vogelstein [Addgene plasmid #16539]) (35). Primers are listed in Table 1. Subsequent ILEI promoter truncation constructs were cloned by PCR using an alternate 5’ KpnI primer and the same 3’ NheI primer, or using Q5 SDM kit from NEB (MA, USA). ILEI 3’UTR construct from stop codon to 1,620 base pairs downstream of the stop codon was amplified by PCR from 501-Mel cDNA with 5’ NheI and 3’ Sall primers and ligated into pmirGLO Dual-Luciferase vector (RAB14 3’UTR WT was a gift from Curt Civin [Addgene plasmid # 61489]). (52) The following constructs were used in this study: pcDNA3-cmyc (a gift from Wafik El-Deiry [Addgene plasmid # 16011) (53), pCDNA3-HA-human MYCN (a gift from Martine Roussel [Addgene plasmid # 74163] (54), pMXs-Hu-L-Myc (a gift from Shinya Yamanaka [Addgene plasmid # 26022] (55), pEGFP-N1-TFEB (a gift from Shawn Ferguson [Addgene plasmid # 38119] (56), pEGFP-N1-TFE3 (a gift from Shawn Ferguson [Addgene plasmid # 38120] (56), pCMV-Tag4A-MITF-M (wt) (a gift from Yardena Samuels [Addgene plasmid # 31151]), and USF1, CREB3L2, and ID2 coding sequence constructs in pLX304 were generated by David Root and supplied by DNASU (57-60). Corresponding empty vector for human L-MYC was constructed by digesting the L-MYC plasmid with NotI to remove L-MYC coding sequence and religating using T4 ligase.

**Cell culture conditions**
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The following human melanoma cell lines were used: WM3918, 501-Mel, Sk-Mel-28, WM983B, 1205Lu, and WM9. These cell lines were purchased from ATCC, Coriell, or were a generous gift from Dr. J. Alan Diehl or Dr. Alain Mauviel. All melanoma cell lines were cultured at 37°, 5% CO2 in RPMI-1640 medium (Hyclone; Logan, UT; USA) supplemented with 10% FBS (Atlanta Biologicals; Flowery Branch, GA; USA), Antibiotic-Antimycotic (100x; ThermoFisher; Waltham, MA; USA), and prophylactic plasmocin (InvivoGen; San Diego, CA; USA) at 37°C and in 5% CO2. HMLE human mammary cell line was obtained from Dr. Sendurai Mani and cultured in DMEM:F12 supplemented with 5% calf serum, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 20 ng/ml EGF and 1% antibiotic/antimycotic.

Stable cell lines were generated by lentiviral transduction with polybrene (8 μg/ml; Sigma-Aldrich; St. Louis, MO; USA). 24h post-transduction the media was changed, and 48h post-transduction the cells were selected and cultured with 0.125 - 0.5 μg/ml puromycin (InvivoGen; San Diego, CA; USA). Pools of stably transduced cells were analyzed. shRNA sequences are listed in Table 2.

UV treatments were conducted using Fisher Scientific UV Crosslinker FB-UVXL-1000 at 0, 5, or 50 J/m2 in room air, and then incubating in normal cell culture conditions for 24h as indicated.

**Lentivirus**

Lentiviral shRNAs were obtained from the MUSC Hollings Cancer Center shRNA Shared Resource Technology. All vectors used in this study are listed in Table 2. Lentivirus was generated by seeding 293T (1,000,000 cells; Takara Bio; Mountainview, CA; USA) to a 60 mm cell culture dish, and transfecting with 6 μL Lipofectamine 2000 (ThermoFisher; Waltham, MA; USA), 1 μg pLKO vector, 0.75 μg psPAX2, and 0.25 μg pMD2.G. 24h post-transfection the media was changed, and 48h and 72h post-transfection the media was harvested. Viral supernatant was cleared by centrifugation, filtered through 0.22 μm filter, and stored at -80° until use.

**Luciferase analysis**

Cell lines were seeded at 50,000 - 75,000 cells per well in 24-well plate in 0.5 ml of complete medium. At 24h the cells were transfected using X-tremeGene 9 (Roche; Switzerland; 100 ng firefly experimental luciferase, 5 ng renilla control luciferase, 0.3 μl X-tremeGENE reagent, in 10 μl Opti-MEM; 200 ng experimental plasmid where indicated [ie – USF1 overexpression construct], 0.6μl X-tremeGENE reagent, in 20 μl Opti-MEM). At this time the cells were treated with DMSO vehicle or 1 μM vemurafenib. At 24h post-transfection the cells were harvested with passive lysis buffer and analyzed with Dual-Glo Luciferase Assay System (Promega; Madison, WI; USA).

**Immunoblot analysis.**

Whole cell lysates were extracted as follows: 100 μL of Tris-Triton lysis buffer (20 mM Tris pH 7.5, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, and Halt Protease and Phosphatase Inhibitor cocktail [ThermoFisher; Waltham, MA; USA]) was added to 6 well cell culture plates, cells were immediately scraped, incubated on ice for 30 minutes, and cleared by centrifugation for 20 minutes at 16,000 x g. Protein concentrations were measured with Bradford Protein Assay (BioRad; Hercules, CA; USA). For conditioned medium immunoblots, cells were serum starved in RPMI/0% FBS overnight, medium was harvested, and precipitated using trichloroacetic acid/acetone. Protein samples were denatured by incubating at 95° for 5 minutes with 1x Laemmli Reducing Denaturating Sample Buffer (300 mM Tris-Cl pH 6.8, 10% SDS, 50% glycerol, 25% BME). 1 – 20 μg of whole cell lysate was resolved on an 8, 10, or 12% polyacrylamide SDS gel, and transferred onto PVDF membrane. Membranes were blocked for 1h at RT in 5% skim milk/Tris-buffered saline with 0.01% Tween-20 (TBST) and incubated overnight at 4° on primary antibody + 5% skim milk/TBST. The following primary antibodies were used: ILEI (ab72182; Abcam; Cambridge, MA; USA; 1:1,000; specificity for the band between 25 and 20 kDa confirmed in (25)), α-tubulin (2144; Cell Signaling; Danvers, MA; USA; 1:10,000), p-
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ERK T202/Y204 (4370; Cell Signaling; Danvers, MA; USA; 1:2,000), Total ERK (9120; Cell Signaling; Danvers, MA; USA; 1:1000), USF1 (C-20; sc-229; Santa Cruz; Dallas, TX; USA; 1:1,000), GAPDH (sc-2233; Santa Cruz; Dallas, TX; USA; 1:10,000), HSP90 (sc-13119; Santa Cruz; Dallas, TX; USA; 1:10,000). After primary antibody incubation, membranes were washed 4x 15 minutes in TBST and incubated for 1h at RT with secondary antibody in TBST. The following secondary antibodies were used: Goat anti-Mouse IgG (31430; ThermoFisher; Waltham, MA; USA; 1:10,000) and Goat anti-Rabbit IgG3 (31460; ThermoFisher; Waltham, MA; USA; 1:10,000). After secondary antibody incubation, membranes were washed 4x 15 minutes in TBST and detected using Luminata Fast Western HRP substrate (EMD Millipore; Darmstadt, Germany) and HyBlot CL Autoradiography Film (Denville; Holliston, MA; USA) or CCD camera (BioRad ChemiDoc System; BioRad, Hercules, CA, USA).

PCR analysis

Total RNA was isolated using Trizol (Thermo Fisher Scientific; Waltham, MA; USA). Reverse transcription was performed using oligo dT primers and M-MuLV Reverse Transcriptase (New England BioLabs, Ipswitch, MA; USA). Semi-quantitative PCR was conducted on 10 ng of cDNA using Maxima Hot Start PCR Master Mix (Thermo Fisher Scientific; Waltham, MA; USA). Real-time quantitative PCR was conducted using iQ SYBR Green Supermix (BioRad; Hercules, CA; USA) using CFX384 Real-Time System (BioRad; Hercules, CA; USA). Reactions were conducted on 50 pg - 10 ng cDNA. Primers are listed in Table 1. Relative gene expression was calculated using RFX Manager software, and genes were normalized to GAPDH internal control.

Chromatin-Immunoprecipitation (ChIP)

ChIP protocol was modified from Carey et al (61). Briefly, 1.5 x 10^7 cells were fixed in 1% formaldehyde for 10 minutes at room temperature and quenched in 125 mM glycine. Cells were harvested in lysis buffer (5 mM PIPES [pH 8], 85 mM KCl, 0.5% NP-40), and centrifuged for 10 min (3,000 rpm, 4°).

Supernatant (cytosolic fraction) was removed, and pellet was resuspended in nuclei lysis buffer (50 mM Tris [pH 8], 10 mM EDTA, 1% SDS, protease and phosphatase inhibitor tab). Nuclear extracts were sonicated for 10 min (30 sec on - 30 sec off), and cleared by centrifugation (2x, 10 min, 13,000 rpm, 4°). The supernatant was considered the chromatin fraction. 100 μg of chromatin samples were precleared for 2h at 4° with 30 μl slurry ChIP-Grade Protein G Agarose Beads (9007; Cell Signaling; Danvers, MA; USA). Resulting supernatants were incubated overnight on a rotator at 4° with 5 μg of control Mouse (G3A1) mAb IgG1 Isotype Control (5415; Cell Signaling; Danvers, MA; USA) or USF1 antibody (C-20). 30 μl slurry ChIP-Grade Protein G Agarose Beads was added to each sample for 2h on a rotator at 4°. Beads were washed 4x in high-salt wash buffer (50 mM HEPES [pH 7.9], 500 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 0.1% deoxycholate) each time incubating for 10 min on a rotator at room temperature. Beads were next washed 2x in TE buffer each time incubating for 10 min on a rotator at room temperature. Beads were resuspended in 300 μl elution buffer (50 mM Tris [pH 8], 10 mM EDTA, 1% SDS) + 20 μg RNase A (R1253; Thermo Fisher Scientific; Waltham, MA; USA) and incubated on a 55° heat block for 2h. 1.6 mU of proteinase K (P8107S; New England BioLabs; Ipswitch, MA; USA) were added prior to another incubation on a 55° heat block for 2h. The samples were transferred to a 65° heat block for overnight elution. The samples were purified in 30 μl H2O using GeneJET PCR Purification Kit (K0702; Thermo Fisher Scientific; Waltham, MA; USA). PCR was conducted with primers used for FAM3C promoter cloning specific for human FAM3C promoter from 300 bp to 80 bp upstream of TSS, listed in Table 1), human TYR promoter or human HMOX1 promoter (15,42).

Biotin DNA pulldown

DNA pulldown assays were conducted with 5'-biotinylated double-stranded annealed oligonucleotides corresponding to FAM3C promoter from -200 to -110 upstream of the TSS. A mutant E-box construct was used in which the E-box at -162 to -156 was mutated.
USF-1 regulates ILEI transcription from CACGTG to CAAATG. A 5’-biotinylated double-stranded annealed oligonucleotide of a random sequence was used as a negative control (SCR).

Nuclear extracts were isolated by harvesting cells in lysis buffer (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.05 % NP40, pH 7.9, protease and phosphatase inhibitor tab) and pelleting cell nuclei. Pellets were incubated for 30 min on ice in nuclei extraction buffer (5 mM HEPES, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol, pH 7.9, protease and phosphatase inhibitor) and NaCl added to a final concentration of 300 mM. Samples were homogenized by passing through a 28 g needle 20 times. Samples were incubated on ice for 30 min and centrifuged. The supernatants were considered the nuclear extract. Nuclear extract (20 µg) was reserved as 5% input.

Preclear beads were prepared as follows: 5’-biotinylated random oligonucleotide (SCR) was bound to streptavidin agarose beads (50 µl slurry + 1 µg oligo; ThermoFisher; Waltham, MA; USA) for 1h on a rotator at room temperature. Preclear was conducted as follows: 400 µg nuclear extract was incubated with preclear beads for 1h on a rotator at room temperature. The resulting sample was centrifuged and the supernatant was precleared again for a total of three preclears. The final supernatant was incubated on IP beads overnight on a rotator at 4°. The beads were washed in PBS/0.1% Triton X-100 and resolved by SDS-PAGE, transferred to nitrocellulose, and probed using antibody against USF1 (C-20; sc-229; Santa Cruz; Dallas, TX; USA; 1:1,000).

Statistical analyses

Data are mean +/- standard deviation unless indicated otherwise. p < 0.05 by unpaired two sample Student’s T-test is considered significant. Representative experiments are repeated at least twice.

Wound healing assays

Cells (3x 10⁵; 0.5 ml of complete medium) were seeded in a 24 well plate, and a 1 ml pipette tip was used to scratch the cells. Images were recorded from 0 to 24 hours, and analyzed using ImageJ (NIH; Bethesda, MD; USA).

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Tables.

Table 1: Primer sequences.

| Primer name                        | Sequence                              |
|-----------------------------------|---------------------------------------|
| Cloning -150 KpnI ILEI promoter F | GGC GGT ACC AAG TTC AGA TTG TGC AGC G |
| Cloning -204 KpnI ILEI promoter F | GGC GGT ACC ACG TGG CAA GTT CAG ATT  |
| Cloning -221 KpnI ILEI promoter F | GGC GGT ACC CAT TT TCT CCC TCC CGT AGI|
| Cloning -300 KpnI ILEI promoter F | GGC GGT ACC ATG GGAT GGG TCA TTT AAA ATG TTC TGC |
| Cloning -2303 KpnI ILEI promoter F| GGC GGT ACC GGA TTC TCC AAA TAC TCC ATC AGT G |
| Cloning -80 NheI ILEI promoter R  | TTA GCT AGC AGG GCC CGG AGA GCG GA    |
| SDM E-box ILEI promoter F         | CGG CAC TCC CCA AAT GCC AGG TTC AGA TTG TGC AGC GCC TGG C |
| SDM E-box ILEI promoter R         | CCG CGG CAG CGG CCC TGC                |
| SDM Inr ILEI promoter F           | GTA AAA AAA GTG CAG CGC CTG GCC GGG   |
| SDM Inr ILEI promoter R           | TTG CTT TTT TGG AGC TGC CGC CGC CGC A |
| Cloning NheI ILEI 3’ UTR F       | ATT GCT AGC TGG AAA TGT GGA GAG AAT TGA AG |
| Cloning ILEI 3’ UTR SalI R       | TTA GTC GAC CTA CAA CAT TTA TTT CAC AAT CCC T |
| RT-PCR ACTB F                     | ATG CTT CTA GCC GGA CTA TG            |
| RT-PCR ACTB R                     | ACA AAT AAA GCC ATG CAA AT            |
| RT-PCR FAM3C F                    | GCA ACC AAA CTC AAT GAT GA            |
| RT-PCR FAM3C R                    | ACC ACA GAA GAC CCA GTC AT            |
| RT-PCR FAM3C Intron F             | TTG CCCTAA TGC AGATCA TA              |
| RT-PCR FAM3C Intron R             | CAA CAA AGA AAC CCA CAA CA           |
| RT-PCR GAPDH F                    | CTC CTC ACA GGT GCC ATG TA           |
| RT-PCR GAPDH R                    | GGT TGA GCA CAG GGT ACT TT           |
| RT-PCR TYR F                     | ACC TCT CAT TTG CAA GGT CAA A        |
| RT-PCR TYR R                     | AGG AGG AAC CTC TGC CGA AAA GC       |
| RT-PCR USF1 F                    | GCA CTC GTC AAT TCT TTG TG           |
| RT-PCR USF1 R                    | TTC TGA CTT CGG GGA ATA AG           |

Table 2: shRNA sequences.

| shRNA name                        | Target   | Sequence                              |
|-----------------------------------|----------|---------------------------------------|
| pLKO.1-puro Non-mammalian shRNA   | Scrambled| CCGGCAACAAGATGAAGAGCACCACCAA CTCTGCAGTTCTGGTCTCTC CATCTCTTGGTT GTTTTT |
| TRC2-pLKO-puro TRCN000020679      | USF1     | CCGGGCTGGATACTGGACACACTAA CTCAGTTGGATGCTGGATCCAGTATCCAG CTTTTT |
| TRC2-pLKO-puro TRCN000020681      | USF1     | CCGGCACTGGTCAATTCTTTTGTGATC TCGAGATACAAAGAATGACCAGT GTTTTT |
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Figures.

Fig 1. ILEI regulates lung colonization but not primary tumor growth.
USF-1 regulates ILEI transcription

**Fig 1. ILEI regulates lung colonization but not primary tumor growth.** A. Flank injection of 1205Lu cells expressing shSCR or shILEI. N = 5, bar indicates mean +/- SD, n.s. indicates p > 0.05 by Student’s t-test as compared to shSCR. B. Primary tumors from flank injection experiments. C. Tail vein injection of 1205Lu cells expressing shSCR or shILEI. N = 9 for shSCR and N = 7 for shILEI, bar indicates mean +/- SD, p-value indicated by Student’s t-test as compared to shSCR. D. Representative images of H&E stained lung nodules from either shSCR or shILEI.
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Fig 2. Vemurafenib inhibits ILEI expression.

A

WM983B

Vem (h) 0 2 6 24 48

IB: ILEI

IB: p-ERK T202/Y204

IB: Total ERK

IB: GAPDH

B

WM983B

Vem (h) 0 2 6 24 48

PCR: ILEI total RNA

PCR: ILEI pre-RNA

PCR: Actin

C

BRAF WT WM3918

Vem (µM) 0 0.1 0.5 1 5

IB: ILEI

IB: p-ERK T202/Y204

IB: Tubulin

IB: Total ERK

D

Relative ILEI mRNA

0 0.4 0.8 1.2 1.6

DMSO □ Vem

p = 0.04

p = 0.01

1205Lu 501-Mel WM3918

BRAF V600E BRAF WT

E

WM9

Vem - +

IB CM: ILEI

IB: p-ERK T202/-Y204

IB: Total ERK

F

WM9

U0126 (h) 0 24 48

IB: ILEI

IB: p-ERK T202/Y204

IB: Total ERK

IB: GAPDH

PCR: ILEI total RNA

PCR: Actin

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USF-1 regulates ILEI transcription

**Fig 2. Vemurafenib inhibits ILEI expression.** **A.** Immunoblot analysis of ILEI, p-ERK, ERK, and GAPDH levels in WM983B melanoma cell lines treated for 0 to 48 h with vemurafenib (1 µM). **B.** RT-PCR analysis of ILEI, ILEI intron indicates that PCR primers targeted the pre-RNA but not mRNA of the ILEI gene, and Actin levels in WM983B melanoma cell lines treated for 0 to 48 h with vemurafenib (1 µM). **C.** Immunoblot analysis of ILEI, p-ERK, ERK, and Tubulin levels in WM983B melanoma cell lines treated for 24 h with vemurafenib (0-5 µM). **D.** Bar diagram showing quantitative RT-PCR analysis of ILEI levels in 501-Mel, 1205Lu, or WM3918 melanoma cell lines treated for 24 h with vemurafenib (1 µM). **E.** Immunoblot analysis of ILEI, p-ERK, ERK levels in WM9 melanoma cell lines treated for 0 or 24 h with vemurafenib (1 µM). IB CM indicates that serum-free medium condition for 24 h during vemurafenib treatment was harvested and TCA precipitated for immunoblot analysis. **F.** Immunoblot and RT-PCR analysis of ILEI, p-ERK, ERK, GAPDH, and actin levels in WM9 melanoma cell lines treated for 0 to 48 h with U0126 (MEKi, 10 µM).
USF-1 regulates ILEI transcription

Fig 3. Vemurafenib inhibits ILEI expression at the transcriptional level.
USF-1 regulates ILEI transcription

Fig 3. Vemurafenib inhibits ILEI expression at the transcriptional level. A. Design of ILEI promoter reporter construct. The promoter reporter construct spans 2300 base pairs upstream of the TSS to 80 base pairs upstream of the TSS. B. Luciferase assay of ILEI promoter reporter construct in 501-Mel melanoma cell lines treated for 24h with vemurafenib (BRAFi, indicated concentration). N=3, mean +/- SD, * indicates p<0.01 by Student’s T-test as compared to vehicle treatment. C. Luciferase assay of ILEI promoter reporter construct in 501-Mel melanoma cell lines treated for 24h with U0126 (MEKi, indicated concentration). N=3, mean +/- SD, * indicates p<0.01 by Student’s T-test as compared to vehicle treatment. D. Luciferase assay of ILEI promoter reporter construct in 501-Mel melanoma cell lines treated for 24h with U0126 (MEKi, indicated concentration). N=3, mean +/- SD, * indicates p<0.01 by Student’s T-test as compared to vehicle treatment. E. Design of ILEI 3’-UTR reporter construct. The 3’-UTR reporter construct spans from the end of the coding sequence to 1620 bp downstream. F. Luciferase assay of ILEI 3’-UTR reporter construct in 501-Mel melanoma cell lines treated for 24h with vemurafenib (indicated concentration). N=3, mean +/- SD, n.s. indicates p>0.05 by Student’s T-test as compared to vehicle treatment.
USF-1 regulates ILEI transcription

**Fig 4. Proximal E-box contributes ILEI promoter activity.**

**A.** Luciferase assay of various length ILEI promoter reporter constructs in 501-Mel melanoma cell lines. N=3, mean +/- SD, p-value indicated by Student’s T-test as compared to the empty promoterless reporter.

**B.** Luciferase assay of wild-type and E-box mutant (CACGTG to CAAATG) ILEI promoter reporter constructs (-2300/-80) in 501-Mel melanoma cell lines. Black bars indicate wild-type and white bars E-box mutant. N=3, mean +/- SD, p-value indicated by Student’s T-test comparing E-box mutant to wild-type.

**Fig 4. Proximal E-box contributes ILEI promoter activity.** A. Luciferase assay of various length ILEI promoter reporter constructs in 501-Mel melanoma cell lines. N=3, mean +/- SD, p-value indicated by Student’s T-test as compared to the empty promoterless reporter. B. Luciferase assay of wild-type and E-box mutant (CACGTG to CAAATG) ILEI promoter reporter constructs (-2300/-80) in 501-Mel melanoma cell lines. Black bars indicate wild-type and white bars E-box mutant. N=3, mean +/- SD, p-value indicated by Student’s T-test comparing E-box mutant to wild-type.
USF-1 regulates ILEI transcription

Fig 5. Proximal E-box contributes ILEI promoter vemurafenib responsiveness. 

A. Luciferase assay of various length ILEI promoter reporter constructs in 501-Mel melanoma cell lines. Black bars indicate control DMSO treatment and white bars indicate 24h treatment with vemurafenib (1 µM). Values are normalized to DMSO treatment of the particular reporter construct. N=3, mean +/- SD, p-value indicated by Student’s T-test as compared to vemurafenib treatment of the empty promoterless reporter. 

B. Luciferase assay of wild-type and E-box mutant (CACGTG to CAAATG) ILEI promoter reporter constructs (-300/-80) in 501-Mel melanoma cell lines. Black bars indicate control DMSO treatment and white bars indicate 24h treatment with vemurafenib (1 µM). N=3, mean +/- SD, p-value indicated by Student’s T-test comparing vemurafenib to DMSO treatment.
USF-1 regulates ILEI transcription

Fig 6. USF-1 directly regulates ILEI transcription. A. Luciferase assay of ILEI promoter reporter construct (-300/-80) in 501-Mel melanoma cell lines. 0, 0.05, 0.1, 0.2, 0.4 µg of experimental vector and a corresponding 0.4, 0.2, 0.15, 0.1, or 0 µg of empty vector was transfected for 24h. The increased darkness of the bars indicates increased experimental vector. Luminescence is normalized to vector control for each transcription factor. N=3, mean +/- SD, * indicates p<0.01 by Student’s T-test as compared to vector transfection. B. ILEI promoter sequence from -189 to -125 highlighting E-box and initiator (Inr) motifs. C. Various ILEI promoter luciferase reporter constructs either wild-type or mutant for the E-box or the Inr. D. Luciferase assay of ILEI promoter reporter constructs in 501-Mel
USF-1 regulates ILEI transcription in melanoma cell lines. Black bars indicate vector overexpression and white bars indicate USF-1 overexpression. N=3, mean +/- SD, p-value indicated by Student’s T-test as compared to vector.
USF-1 regulates ILEI transcription

Fig 7. USF-1 interacts with the ILEI promoter sequence.

**A**

-200 WT -110
Biotin

WT E-box (CACGTG)

Biotin

-200 mut -110

mut E-box (CAAATG)

Biotin pulldown

Input 5%

SCR WT E-box mut E-box

45 kDa

IB: USF1

**B**

-204

PCR product

TSS

E-box

ILEI promoter

PCR analysis conducted with primers targeting FAM3C, TYR, or HO1 promoter.

Fig 7. USF-1 interacts with the ILEI promoter sequence. A. 5'-biotin tagged ILEI promoter oligonucleotide constructs with wild-type or mutant E-box. Biotin pulldown analysis of nuclear extracts from 501-Mel melanoma cells, followed by immunoblot for USF-1. B. PCR primers flanking ILEI promoter E-box used in ChIP analysis. ChIP analysis of 501-Mel melanoma cell lines immunoprecipitated with control IgG or USF-1 antibody. PCR analysis conducted with primers targeting FAM3C, TYR, or HO1 promoter.
Fig 8. USF-1 in vemurafenib-regulated ILEI expression. A. Biotin pulldown analysis of nuclear extracts from DMSO or vemurafenib treated 501-Mel melanoma cells, followed by immunoblot for USF-1. B. qPCR analysis of USF-1 in Sk-Mel-28 melanoma cells treated with DMSO or vemurafenib (24h, 0 µM). C. Luciferase assay of wild-type ILEI promoter reporter construct (-300/-80) in 501-Mel melanoma cell lines with vector or USF-1 overexpression and DMSO or vemurafenib treatment (1 µM). Black bars indicate DMSO and white bars indicate vemurafenib. N=3, mean +/- SD, p-value indicated by Student’s T-test as compared to DMSO treatment. D. PCR analysis of ILEI, USF-1, and GAPDH in 501-Mel melanoma cell lines overexpressing vector or USF-1 and treated with vemurafenib (24h, 5 µM).
USF-1 regulates ILEI transcription

**Fig 9. Effect of endogenous USF-1 on ILEI expression.**

**A.** PCR analysis for ILEI, TYR, or GAPDH of 501-Mel melanoma cells 24h after treatment with 0, 5, or 50 J/m² of UV.

**B.** qPCR analysis of ILEI in 501-Mel melanoma cells 24h after treatment with 0, 5, or 50 J/m² of UV. N = 3, mean +/- SD, * indicates p < 0.05 compared to 0 UV by Student’s t-test.

**C.** qPCR analysis of ILEI in HMLE human mammary epithelial cells 24h after treatment with 0 or 50 J/m² of UV. N = 3, mean +/- SD, * indicates p < 0.05 compared to 0 UV by Student’s t-test.

**D.** Immunoblot analysis of ILEI, USF-1, or HSP90 in 501-Mel melanoma cells transduced with lentivirus containing scrambled shRNA or two different sequences targeting *USF1*.
USF-1 regulates ILEI transcription

FIG 10. Biological role of USF-1/ILEI axis in melanoma cells.

Fig 10. Biological role of USF-1/ILEI axis in melanoma cells. A. Representative images of wound healing assays of 501-Mel melanoma cells transduced with lentivirus containing scrambled shRNA or two different sequences targeting USF1. B. Quantification of panel A using ImageJ software. N = 3, mean +/- SEM, and * indicates p < 0.05 compared to scrambled by unpaired Student’s t-test.
USF-1 regulates ILEI transcription

**FIG 11. Model of USF-1 regulation of ILEI.**

Model depicting basal vs. vemurafenib-treated conditions. BRAFi: B-Raf kinase inhibitor. ILEI: Interleukin-like EMT Inducer, FAM3C. TSS: Transcriptional start site. USF-1: Upstream stimulatory factor-1. Vem: Vemurafenib.
Interleukin-like EMT inducer (ILEI) promotes melanoma invasiveness and is transcriptionally up-regulated by upstream stimulatory factor-1 (USF-1)
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