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Master transcription factors form interconnected circuitry and orchestrate transcriptional networks in esophageal adenocarcinoma

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Abstract

Objectives: While esophageal squamous cell carcinoma (ESCC) remains infrequent in Western populations, the incidence of esophageal adenocarcinoma (EAC) has increased 6- to 8-fold over the past 4 decades. We aimed to characterize esophageal cancer- and subtypes-specific gene regulation patterns and their upstream transcription factors (TFs).

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Author Contributions

D.-C.L. conceived and devised the study. D.-C.L., L.C. and M.H designed experiments and analysis. L.C., J.P., J.P., Y-Y.J., S.C., L-W.D., J.W.S., N.D. performed the experiments. M.H., T.C.S., O.A., Q.Y., H.Y., B.P.B., performed bioinformatics and statistical analysis. Y.C., W.N.M.D., K.W., R.T., S.A.G., S.J.K. and S.J.M. contributed reagents and materials. L.C., M.H., J.P., D.-C.L., and H.P.K. analyzed the data. D.-C.L. and H.P.K. supervised the research and wrote the manuscript.

Conflict of interest statement

The authors declare no potential conflicts of interest.

Design: To identify regulatory elements, we profiled fresh-frozen esophageal normal samples, tumors and cell lines with chromatin immunoprecipitation sequencing (ChIP-Seq). Mathematical modeling was performed to establish (super)-enhancers landscapes and inter-connected transcriptional circuitry formed by master TFs. Co-regulation and cooperation between master TFs were investigated by ChIP-Seq, 4C-Seq and luciferase assay. Biological functions of candidate factors were evaluated both in vitro and in vivo.

Results: We found widespread and pervasive alterations of the (super)-enhancer reservoir in both subtypes of esophageal cancer, leading to transcriptional activation of a myriad of novel oncogenes and signaling pathways, some of which may be exploited pharmacologically (e.g., LIF pathway). Focusing on EAC, we bioinformatically reconstructed and functionally validated an interconnected circuitry formed by 4 master TFs: ELF3, KLF5, GATA6 and EHF, which promoted each others' expression by interacting with each super-enhancer. Downstream, these master TFs occupied almost all EAC super-enhancers and cooperatively orchestrated EAC transcriptome. Each TF within the transcriptional circuitry was highly and specifically expressed in EAC and functionally promoted EAC cell proliferation and survival.

Conclusions: By establishing cancer- and subtype-specific features of EAC epigenome, our findings promise to transform understanding of the transcriptional dysregulation and addiction of EAC, while providing molecular clues to develop novel therapeutic modalities against this malignancy.

Keywords

Transcription factor; gene regulation; signal transduction; esophageal cancer

INTRODUCTION

Transcriptional dysregulation is a prominent hallmark of cancer. The cis-regulatory elements known as enhancers are key modulators of cell type-specific expression programs. Recently, a unique group of enhancers, termed super-enhancers, has been identified[1]. Super-enhancers recruit an exceptionally large number of transcription factors (TFs) and cofactors and can be identified by extensive active histone marks, such as histone 3 lysine 27 acetylation (H3K27Ac)[1, 2]. Importantly, we[3, 4, 5] and others[6, 7, 8, 9] have shown that malignant transformation is accompanied by locus-specific gains and losses in enhancer activity - particularly super-enhancers - across the epigenome, resulting in widespread changes in transcriptional output. Recent studies have suggested that a small number of TFs are critical for orchestrating specific gene expression programs by regulating most cell-specific super-enhancers[1, 10]. These TFs, called "Master" TFs, are often associated with super-enhancers themselves, and control their own transcription and that of other master TFs through an interconnected auto-regulatory circuitry[11, 12, 13]. This transcriptional paradigm is exemplified in embryonic stem cells, wherein master TFs (OCT4, SOX2 and NANOG) bind to their own and each other's super-enhancers, forming an interconnected circuitry[11]. In squamous cell carcinomas, our group has recently characterized δNp63 and SOX2 as master TFs that form an interconnected circuitry[14].

As the eighth most common cancer and sixth leading cause of cancer-related mortality worldwide[15], esophageal cancer is classified histologically as either adenocarcinoma (EAC) or squamous cell carcinoma (ESCC). While ESCC remains infrequent in Western populations, the incidence of EAC has strikingly increased 6- to 8-fold in Western countries over the past 4 decades[16]. The prognosis of patients with EAC remains very poor, with a 5-year survival rate of 17% in the United States[15]. Many EAC genomic drivers have been identified, including mutations in *TP53, KRAS, CDKN2A, ARID1A, SMAD4* [5, 17, 18, 19, 20, 21], offering new insights into the pathogenesis of this malignancy. However, in stark contrast to genomic alterations, our understanding of the EAC epigenome is largely confined to DNA methylation changes at selected genome loci[22, 23]. Very recently, Britton *et al.* performed ATAC-Seq and identified open chromatin regions in 3 EAC cell lines and 6 EAC tumor samples, revealing important upstream TFs (AP1 and ETS factors)[24]. Nevertheless, EAC-associated cistrome aberrations and their biological significance remain poorly characterized. The current study addressed these crucial questions by comprehensively and integratively analyzing the molecular features of the epigenome of EAC.

Materials and Methods are described in Supplementary Data.

RESULTS

Characterization of enhancer landscapes in esophageal cancer

To establish the landscapes of active regulatory elements in esophageal cancer, we profiled 11 fresh-frozen EAC tumor specimens and 9 well-annotated EAC cell lines using chromatin immunoprecipitation sequencing (ChIP-Seq) of H3K27Ac. To identify subtype-specific features of chromatin modification, we re-processed H3K27Ac ChIP-Seq data from 6 ESCC cell lines that we had generated previously[4, 5, 25] (Supplementary Table 1). After peak identification, we separately explored elements that were either transcriptional start site (TSS)-proximal (within 2kb of TSS, putative active promoters) or TSS-distal (beyond 2kb of any TSS, putative active enhancers), given their different roles in the regulation of gene expression programs. Notably, enhancer peaks exhibited much greater variability between groups of samples than did promoter peaks. For example, with FDR<0.001 and fold-change of peak intensity >4, there were 19,617 differential enhancer peaks when comparing EAC and ESCC samples, 17 times greater than differential promoter peaks (n=1,151). The increased variability in enhancer region was not simply due to the larger number of enhancer elements (n=47,162/sample) compared with promoter elements (n=10,286/sample, Supplementary Table 1). These results suggest pervasive genome-wide alterations in enhancer (but not promoter) activities between EAC and ESCC samples; therefore, we focused on enhancer element for subsequent analyses.

Hierarchical clustering using active enhancers with the most variable intensities (*i.e.,* the top 10,000) clearly separated ESCC and EAC samples (Fig. 1A). We readily reproduced this clustering pattern with a different statistical cutoff when selecting the most variable enhancer signals (using FDR<0.001, Supplementary Fig. 1), demonstrating the robustness of the clustering approach. Moreover, EAC tumor specimens and cell lines clustered together, suggesting a convergent enhancer state markedly distinct from ESCC. This convergence of epigenomic state between tumor samples and cell lines has been observed in other types of

cancers, such as rhabdomyosarcoma[26] and neuroblastoma[27]. The similarity of enhancer landscapes between EAC tumor specimens and cell lines not only suggests that the difference between EAC and ESCC samples is not simply due to cell culture, but also allowed us to utilize cell line models to investigate some of the most important EAC-specific enhancer features, discussed below.

By focusing on protein-coding genes, differential analysis identified 6,537 gained enhancers (assigned to 3,189 genes) and 6,876 lost enhancers (assigned to 3,587 genes) in EAC *vs.* ESCC samples (Fold change>2, FDR<0.001, Supplementary Table 2). Although this differential usage of enhancers is partially contributed by the different cell identity, alterations in enhancer landscapes also clearly reflect subtype-specific cancer biology. Particularly, these subtype-specific enhancers were associated with genes enriched in signaling pathways displaying subtype-specific features (Fig. 1B). For example, EAC-high enhancer genes were over-represented in the HIF-1α, cytokine[28], FOXA1[22], TNF, PDGFR[22] and ARF6[22] pathways, while ESCC-high enhancers were more significantly enriched in Hippo[4, 22], δNp63 [5, 25, 29], Rac1, focal adhesion and NRF2[5, 22, 29, 30, 31] signaling. These data were highly consistent with previous reports characterizing differential activities of signaling pathways between EAC and ESCC (e.g., FOXA1 and ARF6 signaling were stronger in EAC, while Hippo, and NRF2 activities were more elevated in ESCC[22]).

We next investigated whether differences in enhancer landscapes between groups reflected changes in transcriptomic output by reanalyzing RNA-Seq data of EAC (n=88) and ESCC (n=90) samples from The Cancer Genome Atlas (TCGA)[22]. Importantly, genes associated with gained enhancers were expressed at significantly higher levels in the corresponding group (Fig. 1C). We and others have previously shown that active enhancers generally exhibit lower DNA methylation levels compared with inactive enhancers and other silenced chromatin elements[32, 33, 34, 35], implying a dynamic competition between TF binding and DNA methylation. Indeed, gained enhancer elements generally exhibited lower DNA methylation levels in the corresponding group (Fig. 1D).

Considering that the different enhancer profiles between EAC and ESCC were partially attributable to their different cell types, we next performed H3K27ac ChIP-Seq analyses on five frozen samples from non-malignant gastroesophageal junction (NGEJ) which have the same columnar cell type with EAC. Importantly, a total of 1,703 NGEJ-high and 485 NGEJ-low enhancers were identified when compared with EAC (Fold change>2, FDR<0.1, Supplementary Table 3), and hierarchical clustering successfully separated these two types of samples (Supplementary Fig. 1B). Pathway enrichment analysis revealed that EAC-high enhancer-associated genes were over-represented in several oncogenic pathways, including the signaling of EGFR, Wnt and EMT. In contrast, processes specific to gastrointestinal mucin-secreting cells, such as O-linked glycosylation of mucin, ware specifically enriched in the NGEJ-high enhancer set. Moreover, bone morphogenic protein (BMP) signaling, which is important for both normal esophagus development[36] and intestinal metaplasia of GEJ cells[37] was only enriched in NGEJ-high genes (Supplementary Fig. 1C). These results highlight dysregulated enhancer landscape in EAC, which is associated with both

cancer- and subtype-specific transcriptional networks apparently contributing to tumor biology.

Distinct super-enhancer landscapes in EAC and ESCC

We recently showed that super-enhancers play prominent roles in regulating the expression of a key array of oncogenes important for the malignant phenotype of cancer cells [3, 4, 25]. Thus, we next annotated super-enhancers in EAC and ESCC samples using the ROSE method[1, 38] (Figs. 1E-G, Supplementary Tables 4a-b) and revealed that subtype-specific super-enhancers accounted for 55.8% (871/1,561) and 53.8% (803/1,493) of all super-enhancers in EAC and ESCC samples (Fig. 1E), respectively. Notably, unique sets of key oncogenes were associated with subtype-specific super-enhancers, many of which reflected subtype-specific cancer biology. For example, ERBB2, MET, GATA6, ETV6 and HNF1B were associated with EAC-specific super-enhancers; CTTN, FGFR2, TP73 and WNT5A were assigned to ESCC-specific ones (Figs. 1F-G). We similarly found that super-enhancer reservoir were substantially alterred between EAC and NGEJ samples, with only 30.4% (554/1,819) being shared (Supplement Fig. 1D-F, Supplementary Tables 4c).

Identification of interconnected transcriptional circuitry formed by master TFs in EAC

After establishing the landscape of enhancers in EAC, we next sought to determine which upstream TFs control the activity of these regulatory elements. Considerable evidence demonstrates that cell-type specific gene expression programs are dominated by a small number of master TFs in each respective cell type[2, 11, 13]. Master TFs are often associated with super-enhancers themselves and form interconnected auto-regulatory loops (also known as core regulatory circuitry) by binding to each others' super-enhancers[11, 13, 33, 39]. Master TFs are also highly expressed in their corresponding cell types. Taking into account these known biological phenomena, we modified a previously established mathematical method[40] and performed integrative circuitry analysis of EAC samples (See Methods), thereby identifying a small set (n=10) of candidate master TFs (Fig. 2A, Supplementary Fig. 2A), including several TFs with known oncogenic functions, such as GATA6, KLF5, FOXA1 and HES1. Compared with other TFs, these candidate master TFs had higher predicted transcriptional connectivity (defined by the magnitude of enrichment of the binding motif of candidate TFs in super-enhancer regions along the genome; Supplementary Fig. 2B).

We reasoned that in a fully interconnected circuitry, the RNA expression of each member should show strong positive correlation in relevant cell/tissue types. We thus interrogated the TCGA EAC RNA-Seq dataset and noted that the expression of 4 candidates (ELF3, KLF5, GATA6 and EHF) displayed prominently positive correlations with each other (Fig. 2B, Supplementary Fig. 2A; all Pearson correlation coefficients>0.2). These correlations were expectedly observed in stomach adenocarcinomas (STAD) given the known molecular similarity between EAC and STAD[22, 41], but were absent in other cancer types, such as breast cancer (Fig. 2B). Moreover, in a pan-cancer RNA-Seq analysis in both TCGA and Cancer Cell Line Encyclopedia (CCLE) samples, these 4 candidates were in general expressed highly in EAC samples relative to most other tumor types (Supplementary Figs. 3 and 6C). We therefore next focused on characterizing these 4 high-confidence master TFs.

To validate direct transcriptional regulation among these candidates, we first performed ChIP-Seq to map the genome-wide occupancy of these 4 master TFs in Eso26, an EAC cell line. We could not generate high-quality ChIP-Seq data of EHF because of lack of Chip grade antibody. Strikingly, ELF3, KLF5 and GATA6 co-occupied the super-enhancers of all 4 master TFs including themselves (Fig. 2E), forming an interconnected circuitry, as we had predicted. Moreover, the super-enhancer regions of all of these 4 TFs were highly specific to both EAC tumor samples and cell lines, as they were substantially weaker in either ESCC cells or NGEJ samples. Serving as additional controls for EAC cells, we further generated H3K27Ac ChIP-Seq data in two Barrett's esophagus (BE) cell lines (ChTRT and GihTRT), which again exhibited negligible signals when compared with EAC samples (Fig. 2E).

To directly confirm their interconnected transcriptional regulation, each TF was silenced using siRNA. Knockdown of any single TF decreased the expression of the other 3 members (Fig. 2C). However, c-MYC, a well-known oncogenic TF in EAC but not predicted within the transcriptional circuitry, was not affected (Fig. 2C, left panel). Similarly, EVX1, a novel oncogenic TF in EAC cells (Manuscript in Preparation) but a non-master TF, was not consistently regulated by these 4 factors. This interconnected circuitry was further confirmed at the protein level using additional individual siRNAs (Fig. 2D). These results were also verified by shRNA-mediated knockdown (Fig. 2C and Fig. 2D). Together, these data identified and validated an interconnected transcriptional circuitry consisting of 4 master TFs in EAC (Fig. 2F).

Master TFs cooperatively orchestrate the transcriptional network of EAC

To understand the significance of interconnected circuitry in regulation of the EAC transcriptome, we next explored in-depth the cistromes of 3 TFs within the circuitry, namely ELF3, KLF5 and GATA6. As expected, motif analysis found highly significant enrichment of their own recognition sequences within the corresponding ChIP-Seq peaks (Fig. 3A). Notably, the binding motif of each single factor was also strongly enriched in the peaks from the other master TFs (Fig. 3A), suggesting that occupancies of these master TFs lie in close proximity to each other. In contrast, either minimum or no enrichment was observed in non-master TFs in EAC (e.g., E2F1 and TP63). Indeed, along the genome, ELF3, KLF5 and GATA6 exhibited a prominent co-occupancy pattern (Fig. 3C). Furthermore, metagene analysis showed that the distribution of these binding peaks strongly aligned (Fig. 3B), suggesting their functional interplay in EAC cells. To investigate the transcriptional implications of the occupancy of ELF3, KLF5 and GATA6, we correlated their binding profiles with H3K27Ac ChIP-Seq data generated from the same Eso26 cell line and observed prominently enriched H3K27Ac signals adjacent to the regions occupied by these master TFs (Figs. 3B-C). Specifically, the majority of ELF3 (87.1%, 5,702/6,543; $P<2.2e-16$), KLF5 (68.7%, 15,215/22,141; $P<2.2e-16$) and GATA6 peaks (76.6%, 3,375/4,404; $P<2.2e-16$, all Chi-squared Test) were associated with H3K27Ac signals, suggesting that transcriptional activation was associated with the binding of these three TFs. Indeed, transcripts assigned to the binding of any single TF were expressed at significantly higher levels than those assigned to none (Fig. 3D).

We next explored the transcriptional impact of the co-occupancy of the master TFs. Importantly, transcripts bound by all three TFs were expressed at the highest levels (Fig. 3D). Given the prominent co-occupying pattern of ELF3, KLF5 and GATA6 in active regulatory regions, we assigned their binding peaks to either super-enhancer or typical-enhancer elements to gain additional insights into their transcriptional co-operation. Akin to a few master TFs identified in other cell types (*e.g.,* PHOX2B, HAND2 and GATA3 in neuroblastoma[27]), EAC master TFs each occupied a significant proportion of super-enhancers but interacted with only a small fraction of typical-enhancers (Supplementary Fig. 4A). In fact, all of the annotated super-enhancers were occupied by at least one of these three TFs. Notably, this preference of interacting with super-enhancers was even more profound when considering co-occupied regions. Specifically, regulatory elements occupied by more TFs had a higher likelihood of lying within super-enhancers than did typical-enhancers (Fig. 3E). These data demonstrate that master TFs not only interconnect via co-regulation within the circuitry, but also cooperatively regulate gene expression programs by preferentially activating super-enhancers along the genome.

ELF3, KLF5 and GATA6 co-operatively activate the super-enhancer of ELF3

Given the above finding that master TF circuitry preferentially activates super-enhancer elements relative to typical-enhancers, we next focused on characterizing ELF3 super-enhancer loci, because ELF3 itself is a top-ranked master TF and has a massive super-enhancer in EAC samples (Fig. 4B). In contrast, the H3K27Ac modification of these enhancer loci was markedly weaker in ESCC samples (Fig. 4B), suggesting that this is an EAC-specific active chromatin state. Next, we employed circularized chromosome conformation capture (4C) assays to explore the interaction landscape of this ELF3 super-enhancer in Eso26 cells, using its promoter as the 4C bait (Viewpoint). Importantly, by cross-referencing H3K27Ac ChIP-Seq data generated in the same cell line, we successfully identified five enhancer constituents (E1-E5) interacting with the ELF3 promoter (Fig. 4C). Moreover, these 5 regions were always co-occupied by both ELF3 and KLF5 (Fig. 4C). GATA6 also had strong interaction with 2 of these 5 enhancer constituents (E1 and E4), suggesting that the activities of these enhancer elements were under control of the three master TFs. These co-occupancy patterns were validated by ChIP-qPCR in multiple additional EAC cell lines (Supplement Fig.4B). Strikingly, these strong and extensive enhancer-promoter interactions were strictly confined within this super-enhancer window (Fig. 4A), indicating that this cluster of enhancer elements were dedicated to activating the transcription of ELF3 in EAC cells. We subsequently cloned individual constituent enhancer elements into the luciferase reporter vector and observed robust activities of E1 and E4 in different EAC cells (Fig. 4D). Consistently, these reporter activities were not detected in ESCC cells (Fig. 4D). To test direct transcriptional regulation of this super-enhancer on ELF3, we used CRISPR interference system wherein sgRNAs guide dCas9/KRAB complex to suppress targeted cis-regulatory elements[42]. We designed sgRNAs against E1 and E4 because: i) these two enhancer elements exhibited the highest reporter activities and ii) they were the only two regions bound by all three TFs. Importantly, targeting either E1 or E4 significantly reduced the expression of ELF3 (Fig. 4D). The expression levels of the other three master TFs were also decreased, again supporting the interconnected co-regulation between these factors. These results support strong and complex regulation of ELF3 super-

enhancer region, which is EAC specific and controlled by EAC master TFs. In parallel, we performed another 4C assay to characterize enhancer-promoter interactions flanking KLF5 super-enhancer region. Again, we validated that multiple enhancer constituents, co-occupied by master TFs, interacted with KLF5 promoter in EAC cells (Supplementary Fig. 5).

Master TFs have strong pro-growth functions in EAC cells

Considering the prominent roles of the master TFs in controlling EAC transcriptional network, particularly their preference in the regulation of super-enhancers, we hypothesized that these factors are required for the viability and proliferation of EAC cells. To test this, we first focused on the investigation of ELF3, whose functional significance in EAC remains unknown. Importantly, depletion of endogenous ELF3 expression by independent siRNAs markedly reduced cell proliferation (Fig. 5A) and colony growth (Fig. 5B) in different EAC cells, and the results were verified by doxycycline-inducible expression of multiple independent shRNAs (Figs. 5E-F). Fluorescence-activated cell sorting (FACS) analysis showed that silencing of ELF3 increased significantly EAC cell apoptosis (Fig. 5C) and cell cycle arrest at S-phase (Fig. 5D). In xenograft assays, induction of the expression of shRNA against ELF3 potently inhibited EAC xenograft growth in mice (Figs. 5G-I). These data characterize that ELF3, a master TF highly expressed in EAC, has strong pro-tumor functions in this cancer. Prompted by the notable pro-survival and pro-proliferation capacities of ELF3, we next tested the functionality of the other 3 master TFs (KLF5, EHF and GATA6). Importantly, silencing of any of the 3 factors inhibited strongly the proliferation and colony formation of multiple different EAC cell lines but not BE cell lines (Figs. 5J-L, Supplementary Figs. 6A-B).

Up-regulated by master TFs via super-enhancers, LIF promotes EAC growth and migration.

Following the identification and characterization of the upstream master TF circuitry, we next focused on investigating the downstream signaling pathways activated by EAC-specific enhancers, inspired by previous work [3, 4, 7, 43] demonstrating that tumor-specific enhancers converge on activating cancer hallmarks and associated signaling pathways. Among the pathways enriched by EAC-specific enhancers, we were particularly interested in the cytokine-mediated signaling since it was top-ranked in EAC group (Fig. 1D). Careful examination of the overlapping pathway components (n=113, Supplementary Table 5) identified many established pro-tumor factors, including LIF, LYN, SYK, JAK2, IL1B, etc. Among these 113 components, LIF was the highest-ranked super-enhancer-assigned cytokine specific to EAC samples (Supplementary Table 4). Moreover, LIF super-enhancer contained co-binding peaks of ELF3 and KLF5 (Fig. 6A). In contrast, these enhancer elements were much weaker in either ESCC, NGEJ or BE samples (Fig. 6A). Consistently, LIF expression was significantly up-regulated in EAC samples (Supplementary Fig. 7A). Immunohistochemistry (IHC) staining observed that LIF protein was strongly expressed in EAC tumors but not in either NGEJ or normal esophageal squamous samples (Fig. 6G). Importantly, silencing of any of the 4 master TFs markedly inhibited the expression of LIF at both mRNA (Fig. 2C) and protein levels (Fig. 2D), strongly suggesting that these master TFs regulate the transcription of this top-ranked super-enhancer gene.

LIF is a well-established pleiotropic cytokine which regulates the differentiation of hematopoietic and neuronal cells. Interestingly, during preparation of the present manuscript, a report was published associating higher LIF level in the serum of EAC patients with worse response to neoadjuvant therapy[44]. However, its biological functions and associated molecular pathways have not been investigated in EAC. To address this, we first depleted LIF transcript by siRNA in multiple EAC cell lines, and its knockdown drastically impaired EAC cell proliferation and colony growth (Figs. 6C-D). In contrast, silencing LIF produced much weaker effect on ESCC cell proliferation, suggesting its EAC-specific role (Supplementary Fig. 7B). Recently, a steroidal LIF-specific small-molecule inhibitor (EC330) was developed[45, 46] (Fig. 6B). Importantly, this LIF-inhibitor displayed potent anti-neoplastic activity in EAC cells in vitro, with IC_{50} ranging from 28–565 nM (Fig. 6B). Again validating the functional specificity of LIF in EAC cells, EC330 barely showed cytotoxicity against ESCC cells (Fig. 6B). Furthermore, exogenous LIF stimulation prominently enhanced cell migration, which was neutralized by co-exposure to an anti-LIF antibody (Fig. 6E, Supplementary Fig. 7C), confirming the specificity of the results. Lastly, exogenous LIF potently stimulated the phosphorylation of STAT3 and AKT pathways (Fig. 6F). Given that both STAT3[47, 48] and AKT signalings are well-established pro-growth cascades for EAC cells, these data characterize LIF as a key super-enhancer-driven factor, which is up-regulated by EAC master TFs and promotes EAC proliferation and migration.

DISCUSSION

Despite numerous new insights gained from genomic analyses of EAC patients[5, 17, 18, 19, 20, 21], preventive or therapeutic strategies have not substantially improved outcomes. EAC exhibits high inter- and intra-tumor genomic heterogeneity[19, 49, 50], increasing the barriers to exploiting targetable genomic lesions. Clearly, alternative molecular approaches in addition to genomic profiling are required to further decipher EAC pathophysiology for the development of more innovative and effective regimens.

To this end, we performed comprehensive epigenome profiling of EAC tumor samples and cell lines, and contrasted them against ESCC and NGEJ samples. We found widespread and pervasive alterations in EAC enhancer and super-enhancer landscapes, which were strongly associated with cancer-specific and subtype-specific biological states. We identified a myriad of novel EAC-promoting genes as well as oncogenic signaling pathways which may be exploited pharmacologically. Amongst these, cytokine signaling represents a particularly important pathway containing many components associated with either EAC-specific enhancers (such as LYN, JAK2, IL1B) or super-enhancers (such as LIF, LIFR). Importantly, IL1B associated signaling was shown to directly promote EAC development and progression in a transgenic mouse model, wherein additional cytokines (e.g., IL6 and IL8) were also significantly upregulated[28]. Here, we identified and validated LIF as a top-ranked super-enhancer-driven cytokine that is uniquely upregulated by master TFs in EAC samples. LIF has strong pro-tumor functions specifically in EAC but not ESCC cells, which can be suppressed by a specific small-molecule inhibitor. Notably, in addition to LIF, the cytokine signaling pathway has a number of components which may be “druggable” (e.g., JAK2, YES1, SYK), highlighting the power of our integrative approach to discover novel

actionable targets, some of which are under early clinical investigation (e.g., NCT02693535).

We established and functionally validated an interconnected transcriptional circuitry formed by master TFs (ELF3, KLF5, GATA6 and EHF), which orchestrates the dysregulation of EAC transcriptome in a co-operative manner. These master TFs promote the expression of each other by interacting with their super-enhancers. Indeed, their mRNA levels significantly correlate with each other, and are generally high in EAC tumors compared with other forms of human cancers. These master TFs operate in concert and often co-occupy enhancer elements in a co-operative fashion. Notably, these factors favor the regulation of super-enhancers over typical-enhancers, such that virtually all of the super-enhancers annotated in Eso26 cells were bound by at least one of these master TFs. This biased pattern of interacting with super-enhancers over typical-enhancers by master TFs was more conspicuous when considering the co-occupied regions. Transcripts bound by all three TFs were expressed at the highest levels, further supporting cooperation (Fig. 3).

Because of this pivotal role of master TFs in the regulation of EAC transcriptomic network (particularly through controlling super-enhancers), all of them are, not surprisingly, required for the survival and proliferation of EAC cells (Fig. 5). Although GATA6 has been established as a strong oncogene in EAC[51], the other three factors (ELF3, EHF and KLF5) remain hitherto unexplored in this cancer. Notably, GATA6 and KLF5 have been shown to interact with each other and promote both activities in gastric cancer[52], which shares a certain degree of genomic similarity with EAC[22]. Both ELF3 and EHF belong to the E26 transformation-specific (ETS) TF family. Intriguingly, both of them have seemingly opposing roles in cancer biology in different tumor types. For example, ELF3 suppresses the activity of androgen receptor and inhibits the proliferation of prostate cancer cells[53]. In ampullary carcinoma, genomic sequencing suggests ELF3 as a tumor-suppressor[54]. While in hepatocellular cancer, ELF3 has oncogenic activities and promotes cellular malignant phenotypes, suggesting a tissue context specific role[55]. Similarly, EHF also has been observed to have opposite functions depending on different tumor types[56, 57]. We reason that these disparities may be because ELF3 and EHF have different transcriptional co-factors/partners in distinct cell types, which results in their different cistromes and downstream genes. In contrast, KLF5 appears to have a consensus oncogenic role in different cancer types. KLF5 has also been recently identified as a master regulator driven by a super-enhancer in low-grade pancreatic ductal adenocarcinoma[58]. Notably, in addition to being activated epigenetically, *KLF5* exons harbor hotspot oncogenic mutations. Furthermore, the super-enhancer of KLF5 was found to be genetically amplified[59]. Interestingly, we also observed that the KLF5 locus is significantly amplified in TCGA EAC samples (data not shown), supporting the notion that prominent driver genes can be altered in cancer cells through multiple different mechanisms.

In summary, by comprehensively establishing the epigenomic state of EAC and its upstream master regulators and downstream signaling pathways, this work promises to transform our understanding of the transcriptional dysregulation and addiction of EAC, while providing potential future therapeutic strategies against this deadly malignancy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ESCC	Esophageal squamous cell carcinoma
EAC	Esophageal adenocarcinoma
TF	Transcription factor
ChIP-Seq	Chromatin immunoprecipitation sequencing
H3K27Ac	Histone 3 lysine 27 acetylation
NGEJ	Non-malignant gastroesophageal junction
DMEM	Dulbecco's modified Eagle medium
FBS	Fetal bovine serum
TSS	Transcriptional start site
TCGA	The Cancer Genome Atlas
CCLE	Cancer Cell Line Encyclopedia
BE	Barrett's esophagus
4C	Circularized chromosome conformation capture
FACS	Fluorescence-activated cell sorting
IHC	Immunohistochemistry

REFERENCES

1. Whyte WA, Orlando DA, Hnisz D, Abraham BJ, Lin CY, Kagey MH, et al. Master transcription factors and mediator establish super-enhancers at key cell identity genes. Cell 2013;153:307–19. [PubMed: 23582322]
2. Hnisz D, Abraham BJ, Lee TI, Lau A, Saint-Andre V, Sigova AA, et al. Super-enhancers in the control of cell identity and disease. Cell 2013;155:934–47. [PubMed: 24119843]
3. Yuan J, Jiang YY, Mayakonda A, Huang M, Ding LW, Lin H, et al. Super-enhancers promote transcriptional dysregulation in nasopharyngeal carcinoma. Cancer Res 2017.
4. Jiang YY, Lin DC, Mayakonda A, Hazawa M, Ding LW, Chien WW, et al. Targeting super-enhancer-associated oncogenes in oesophageal squamous cell carcinoma. Gut 2017;66:1358–68. [PubMed: 27196599]
5. Lin DC, Dinh HQ, Xie JJ, Mayakonda A, Silva TC, Jiang YY, et al. Identification of distinct mutational patterns and new driver genes in oesophageal squamous cell carcinomas and adenocarcinomas. Gut 2017.
6. van Groningen T, Koster J, Valentijn LJ, Zwijnenburg DA, Akogul N, Hasselt NE, et al. Neuroblastoma is composed of two super-enhancer-associated differentiation states. Nat Genet 2017;49:1261–6. [PubMed: 28650485]
7. Mack SC, Pajtler KW, Chavez L, Okonechnikov K, Bertrand KC, Wang X, et al. Therapeutic targeting of ependymoma as informed by oncogenic enhancer profiling. Nature 2018;553:101–5. [PubMed: 29258295]
8. Ooi WF, Xing M, Xu C, Yao X, Ramlee MK, Lim MC, et al. Epigenomic profiling of primary gastric adenocarcinoma reveals super-enhancer heterogeneity. Nat Commun 2016;7:12983. [PubMed: 27677335]
9. Akhtar-Zaidi B, Cowper-Sal-lari R, Corradin O, Saiakhova A, Bartels CF, Balasubramanian D, et al. Epigenomic enhancer profiling defines a signature of colon cancer. Science 2012;336:736–9. [PubMed: 22499810]
10. Lee TI, Young RA. Transcriptional regulation and its misregulation in disease. Cell 2013;152:1237–51. [PubMed: 23498934]
11. Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, et al. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 2005;122:947–56. [PubMed: 16153702]
12. Odom DT, Dowell RD, Jacobsen ES, Nekludova L, Rolfe PA, Danford TW, et al. Core transcriptional regulatory circuitry in human hepatocytes. Mol Syst Biol 2006;2:2006 0017.
13. Odom DT, Zizlsperger N, Gordon DB, Bell GW, Rinaldi NJ, Murray HL, et al. Control of pancreas and liver gene expression by HNF transcription factors. Science 2004;303:1378–81. [PubMed: 14988562]
14. Jiang Y, Jiang YY, Xie JJ, Mayakonda A, Hazawa M, Chen L, et al. Co-activation of super-enhancer-driven CCAT1 by TP63 and SOX2 promotes squamous cancer progression. Nat Commun 2018;9:3619. [PubMed: 30190462]
15. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016;66:7–30. [PubMed: 26742998]
16. Thrift AP, Whiteman DC. The incidence of esophageal adenocarcinoma continues to rise: analysis of period and birth cohort effects on recent trends. Ann Oncol 2012;23:3155–62. [PubMed: 22847812]
17. Dulak AM, Stojanov P, Peng S, Lawrence MS, Fox C, Stewart C, et al. Exome and whole-genome sequencing of esophageal adenocarcinoma identifies recurrent driver events and mutational complexity. Nat Genet 2013;45:478–86. [PubMed: 23525077]
18. Ross-Innes CS, Becq J, Warren A, Cheetham RK, Northen H, O'Donovan M, et al. Whole-genome sequencing provides new insights into the clonal architecture of Barrett's esophagus and esophageal adenocarcinoma. Nat Genet 2015;47:1038–46. [PubMed: 26192915]
19. Secrier M, Li X, de Silva N, Eldridge MD, Contino G, Bornschein J, et al. Mutational signatures in esophageal adenocarcinoma define etiologically distinct subgroups with therapeutic relevance. Nat Genet 2016;48:1131–41. [PubMed: 27595477]

20. Stachler MD, Taylor-Weiner A, Peng S, McKenna A, Agoston AT, Odze RD, et al. Paired exome analysis of Barrett's esophagus and adenocarcinoma. Nat Genet 2015;47:1047–55. [PubMed: 26192918]
21. Agrawal N, Jiao Y, Bettegowda C, Hutfless SM, Wang Y, David S, et al. Comparative genomic analysis of esophageal adenocarcinoma and squamous cell carcinoma. Cancer Discov 2012;2:899–905. [PubMed: 22877736]
22. Cancer Genome Atlas Research N, Analysis Working Group: Asan U, Agency BCC, Brigham, Women's H, Broad I, et al. Integrated genomic characterization of oesophageal carcinoma. Nature 2017;541:169–75. [PubMed: 28052061]
23. Peng D, Hu T, Soutto M, Belkhiri A, Zaika A, El-Rifai W. Glutathione peroxidase 7 has potential tumour suppressor functions that are silenced by location-specific methylation in oesophageal adenocarcinoma. Gut 2014;63:540–51. [PubMed: 23580780]
24. Britton E, Rogerson C, Mehta S, Li Y, Li X, consortium O, et al. Open chromatin profiling identifies AP1 as a transcriptional regulator in oesophageal adenocarcinoma. PLoS Genet 2017;13:e1006879. [PubMed: 28859074]
25. Xie JJ, Jiang YY, Jiang Y, Li CQ, Lim MC, An O, et al. Super-Enhancer-Driven Long Non-Coding RNA LINC01503, Regulated by TP63, Is Over-Expressed and Oncogenic in Squamous Cell Carcinoma. Gastroenterology 2018;154:2137–51 e1. [PubMed: 29454790]
26. Gryder BE, Yohe ME, Chou HC, Zhang X, Marques J, Wachtel M, et al. PAX3-FOXO1 Establishes Myogenic Super Enhancers and Confers BET Bromodomain Vulnerability. Cancer Discov 2017;7:884–99. [PubMed: 28446439]
27. Boeva V, Louis-Brennetot C, Peltier A, Durand S, Pierre-Eugene C, Raynal V, et al. Heterogeneity of neuroblastoma cell identity defined by transcriptional circuitries. Nat Genet 2017;49:1408–13. [PubMed: 28740262]
28. Quante M, Bhagat G, Abrams JA, Marache F, Good P, Lee MD, et al. Bile acid and inflammation activate gastric cardia stem cells in a mouse model of Barrett-like metaplasia. Cancer Cell 2012;21:36–51. [PubMed: 22264787]
29. Song Y, Li L, Ou Y, Gao Z, Li E, Li X, et al. Identification of genomic alterations in oesophageal squamous cell cancer. Nature 2014;509:91–5. [PubMed: 24670651]
30. Lin DC, Hao JJ, Nagata Y, Xu L, Shang L, Meng X, et al. Genomic and molecular characterization of esophageal squamous cell carcinoma. Nat Genet 2014;46:467–73. [PubMed: 24686850]
31. Gao YB, Chen ZL, Li JG, Hu XD, Shi XJ, Sun ZM, et al. Genetic landscape of esophageal squamous cell carcinoma. Nat Genet 2014;46:1097–102. [PubMed: 25151357]
32. Yao L, Shen H, Laird PW, Farnham PJ, Berman BP. Inferring regulatory element landscapes and transcription factor networks from cancer methylomes. Genome Biol 2015;16:105. [PubMed: 25994056]
33. Lin CY, Erkek S, Tong Y, Yin L, Federation AJ, Zapatka M, et al. Active medulloblastoma enhancers reveal subgroup-specific cellular origins. Nature 2016;530:57–62. [PubMed: 26814967]
34. Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, et al. Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 2009;462:315–22. [PubMed: 19829295]
35. Silva TC, Coetzee GS, Yao L, Gull N, Hazelett DJ, Noushmehr H, et al. ELMER v.2: An R/Bioconductor package to reconstruct gene regulatory networks from DNA methylation and transcriptome profiles. bioRxiv 2018;148726.
36. Pavlov K, Meijer C, van den Berg A, Peters FT, Kruyt FA, Kleibeuker JH. Embryological signaling pathways in Barrett's metaplasia development and malignant transformation; mechanisms and therapeutic opportunities. Crit Rev Oncol Hematol 2014;92:25–37. [PubMed: 24935219]
37. Milano F, van Baal JW, Buttar NS, Rygiel AM, de Kort F, DeMars CJ, et al. Bone morphogenetic protein 4 expressed in esophagitis induces a columnar phenotype in esophageal squamous cells. Gastroenterology 2007;132:2412–21. [PubMed: 17570215]
38. Loven J, Hoke HA, Lin CY, Lau A, Orlando DA, Vakoc CR, et al. Selective inhibition of tumor oncogenes by disruption of super-enhancers. Cell 2013;153:320–34. [PubMed: 23582323]

39. Mansour MR, Abraham BJ, Anders L, Berezovskaya A, Gutierrez A, Durbin AD, et al. Oncogene regulation. An oncogenic super-enhancer formed through somatic mutation of a noncoding intergenic element. Science 2014;346:1373–7. [PubMed: 25394790]
40. Huang M, Chen Y, Yang M, Guo A, Xu Y, Xu L, et al. dbCoRC: a database of core transcriptional regulatory circuitries modeled by H3K27ac ChIP-seq signals. Nucleic Acids Res 2017.
41. Hayakawa Y, Sethi N, Sepulveda AR, Bass AJ, Wang TC. Oesophageal adenocarcinoma and gastric cancer: should we mind the gap? Nat Rev Cancer 2016;16:305–18. [PubMed: 27112208]
42. Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell 2013;154:442–51. [PubMed: 23849981]
43. Wang Y, Zhang T, Kwiatkowski N, Abraham BJ, Lee TI, Xie S, et al. CDK7-dependent transcriptional addiction in triple-negative breast cancer. Cell 2015;163:174–86. [PubMed: 26406377]
44. Buckley AM, Lynam-Lennon N, Kennedy SA, Dunne MR, Aird JJ, Foley EK, et al. Leukaemia inhibitory factor is associated with treatment resistance in oesophageal adenocarcinoma. Oncotarget 2018;9:33634–47. [PubMed: 30263091]
45. Nair HB, Santhamma B, Viswanadhapalli S, Sareddy GR, Manthati V, Vadlamudi RK, et al. Discovery and preclinical pharmacology of EC330: A first-in-class leukemia inhibitory factor (LIF) inhibitor. European Journal of Cancer 2016.
46. Nair H, Santhamma B, Viswanadhapa S, Nickisch K. First-in-class steroidal leukemia inhibitory factor (LIF) inhibitor in targeted cancer therapy. American Association for Cancer Research 2016;76:LB-208.
47. Timme S, Ihde S, Fichter CD, Waehle V, Bogatyreva L, Atanasov K, et al. STAT3 expression, activity and functional consequences of STAT3 inhibition in esophageal squamous cell carcinomas and Barrett's adenocarcinomas. Oncogene 2014;33:3256–66. [PubMed: 23912451]
48. Dvorak K, Chavarria M, Payne CM, Ramsey L, Crowley-Weber C, Dvorakova B, et al. Activation of the interleukin-6/STAT3 antiapoptotic pathway in esophageal cells by bile acids and low pH: relevance to barrett's esophagus. Clin Cancer Res 2007;13:5305–13. [PubMed: 17875759]
49. Murugaesu N, Wilson GA, Birkbak NJ, Watkins T, McGranahan N, Kumar S, et al. Tracking the genomic evolution of esophageal adenocarcinoma through neoadjuvant chemotherapy. Cancer Discov 2015;5:821–31. [PubMed: 26003801]
50. Pectasides E, Stachler MD, Derks S, Liu Y, Maron S, Islam M, et al. Genomic Heterogeneity as a Barrier to Precision Medicine in Gastroesophageal Adenocarcinoma. Cancer Discov 2018;8:37–48. [PubMed: 28978556]
51. Lin L, Bass AJ, Lockwood WW, Wang Z, Silvers AL, Thomas DG, et al. Activation of GATA binding protein 6 (GATA6) sustains oncogenic lineage-survival in esophageal adenocarcinoma. Proc Natl Acad Sci U S A 2012;109:4251–6. [PubMed: 22375031]
52. Chia NY, Deng N, Das K, Huang D, Hu L, Zhu Y, et al. Regulatory crosstalk between lineage-survival oncogenes KLF5, GATA4 and GATA6 cooperatively promotes gastric cancer development. Gut 2015;64:707–19. [PubMed: 25053715]
53. Shatnawi A, Norris JD, Chaveroux C, Jasper JS, Sherk AB, McDonnell DP, et al. ELF3 is a repressor of androgen receptor action in prostate cancer cells. Oncogene 2014;33:862–71. [PubMed: 23435425]
54. Yachida S, Wood LD, Suzuki M, Takai E, Totoki Y, Kato M, et al. Genomic Sequencing Identifies ELF3 as a Driver of Ampullary Carcinoma. Cancer Cell 2016;29:229–40. [PubMed: 26806338]
55. Zheng L, Xu M, Xu J, Wu K, Fang Q, Liang Y, et al. ELF3 promotes epithelial-mesenchymal transition by protecting ZEB1 from miR-141-3p-mediated silencing in hepatocellular carcinoma. Cell Death Dis 2018;9:387. [PubMed: 29523781]
56. Albino D, Civenni G, Dallavalle C, Roos M, Jahns H, Curti L, et al. Activation of the Lin28/let-7 Axis by Loss of ESE3/EHF Promotes a Tumorigenic and Stem-like Phenotype in Prostate Cancer. Cancer Res 2016;76:3629–43. [PubMed: 27197175]
57. Cheng Z, Guo J, Chen L, Luo N, Yang W, Qu X. Knockdown of EHF inhibited the proliferation, invasion and tumorigenesis of ovarian cancer cells. Mol Carcinog 2016;55:1048–59. [PubMed: 26258986]

58. Diaferia GR, Balestrieri C, Prosperini E, Nicoli P, Spaggiari P, Zerbi A, et al. Dissection of transcriptional and cis-regulatory control of differentiation in human pancreatic cancer. EMBO J 2016;35:595–617. [PubMed: 26769127]

59. Zhang X, Choi PS, Francis JM, Gao GF, Campbell JD, Ramachandran A, et al. Somatic Superenhancer Duplications and Hotspot Mutations Lead to Oncogenic Activation of the KLF5 Transcription Factor. Cancer Discov 2018;8:108–25. [PubMed: 28963353]

Summary

What is already known about this subject?

- Malignant transformation is associated with gains and losses in enhancer activity across the epigenome, resulting in widespread changes in transcriptional regulation.
- A small number of master TFs are instrumental for orchestrating gene expression programs by regulating cell-specific (super)-enhancers.
- The genomic landscapes of ESCC and EAC have been established and contrasted; however, their epigenomic features have not been comprehensively and systematically compared and analyzed.

What are the new findings?

- EAC and ESCC display distinct (super)-enhancer landscapes, which contribute to subtype-specific transcriptional dysregulation.
- An interconnected transcriptional circuitry in EAC formed by 4 master TFs (ELF3, KLF5, GATA6 and EHF) is identified and validated.
- Master TFs occupy most of EAC super-enhancers and cooperatively orchestrated EAC transcriptome, thereby promoting the survival and proliferation of EAC cells.
- Transcriptionally activated by master TFs through EAC-specific super-enhancers, LIF contributes to the malignant phenotypes of EAC cells.

How might it impact on clinical practice in the foreseeable future?

- This work uncovers many epigenomic features that may help develop novel therapeutic modalities for treating EAC patients. Particularly, EAC-specific super-enhancers activate a number of oncogenes and signaling pathways, some of which may be exploited pharmacologically (e.g., LIF pathway).
- EAC cells are transcriptionally addicted to master TFs (ELF3, KLF5, GATA6 and EHF), and likely their associated transcriptional cofactors, which may offer a novel strategy to fight against this malignancy.

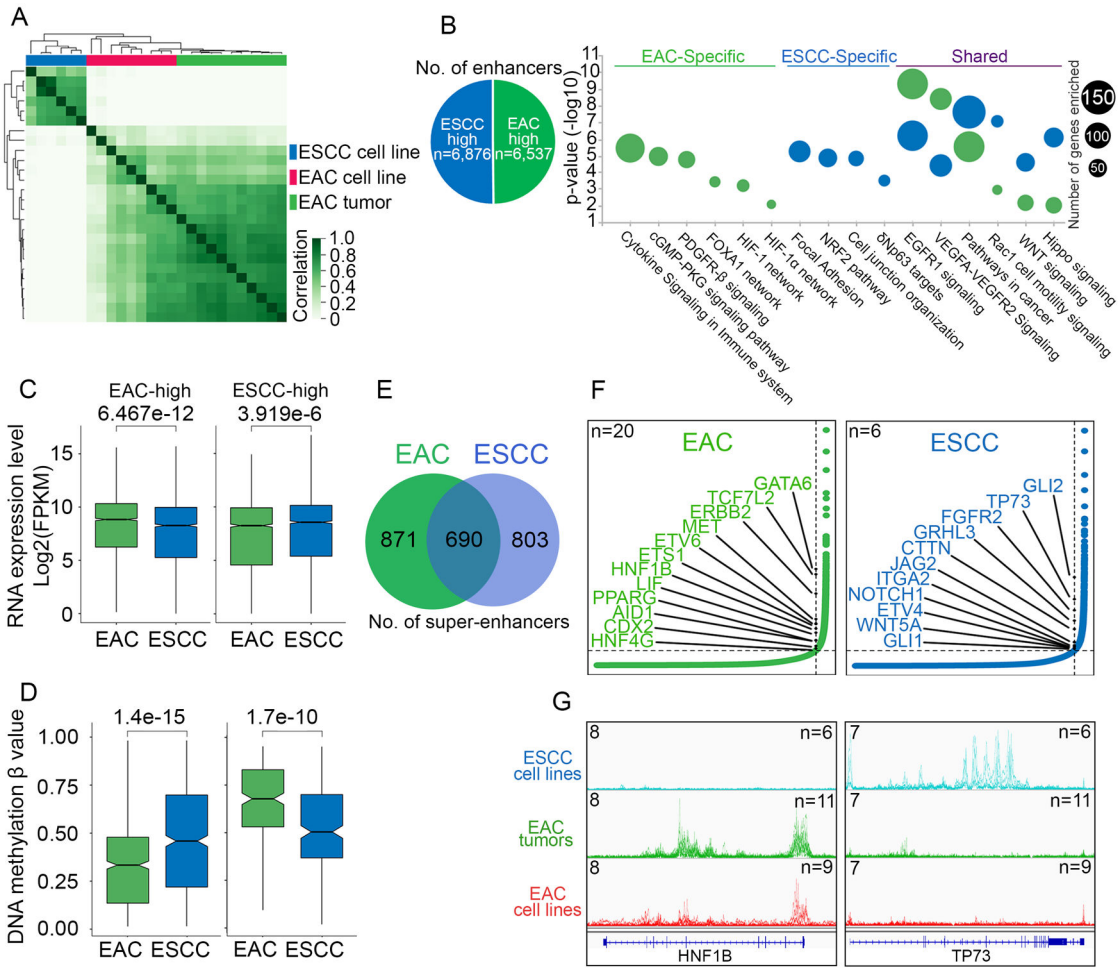

Figure 1. Enhancer and super-enhancer landscapes of esophageal cancers.

(A) Hierarchical clustering using enhancers with the most variable intensities (top 10,000). (B) Left, pie chart showing the number of gained enhancers in each group; Right, pathway enrichment of gained enhancers. Dot size denotes the number of genes enriched. (C) Box plot of mRNA levels of genes associated with changed enhancers in EAC and ESCC samples from TCGA. (D) Box plot of DNA methylation levels of changed enhancer loci in EAC and ESCC samples from TCGA. P value was determined by Wilcox Test. (E) Venn diagram of the number of super-enhancers annotated in each group. (F) Inflection plot ranking enhancer intensities, and only group-specific super-enhancers are displayed as examples. (G) IGV plots of the H3K27Ac ChIP-Seq profiles of group-specific super-enhancers. Each line represents one sample; values of normalized ChIP-Seq signal intensities are shown on the upper left corner; genomic structure of the genes associated with super-enhancer is shown at the bottom.

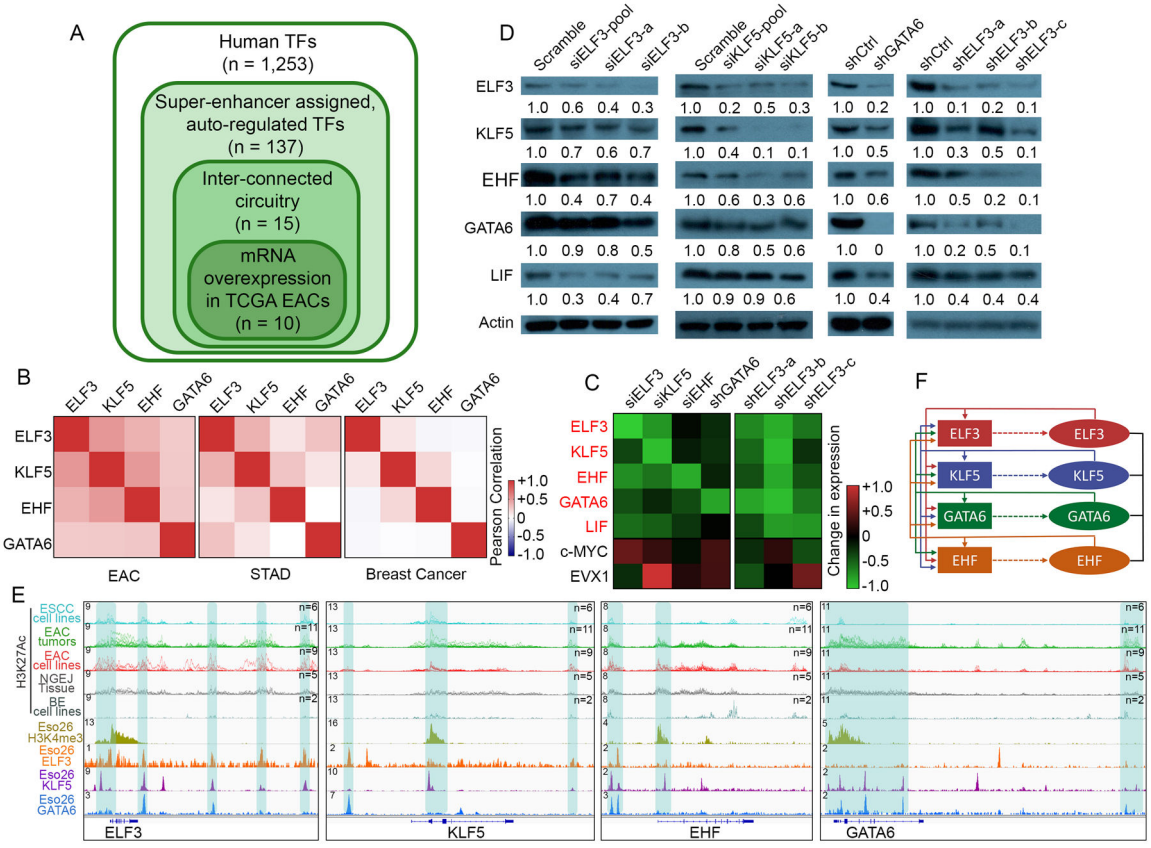

Figure 2. Master TFs form interconnected transcriptional circuitry in EAC.

(A) Integrative methods for identification of candidate master TFs. (B) Heatmap of Pearson correlation coefficient between candidate master TFs in TCGA EAC (n=88), stomach adenocarcinoma (STAD, n=415) or breast cancer samples (n=1,100). (C) Heatmap of fold changes of mRNA levels of master TFs and c-Myc and EVX1 (non-master TFs, negative control) following siRNA knockdown of each master TF (left) or 3 different shRNAs against ELF3 (right). (D) Western Blot validating the co-regulation among master TFs in Eso26 cells. The numbers denote the densitometric quantitation of band intensity, normalized by Actin levels. (E) IGV plot of ChIP-Seq showing co-occupancy (shaded) of ELF3, KLF5 and GATA6 at the super-enhancers of their own gene and the other 3 master TFs. Antibodies against endogenous KLF5 and GATA6 were used. A flag antibody for exogenous ELF3-Flag was used because of the poor quality of ELF3 antibody for ChIP-Seq. (F) Schematic graph of the model of interconnected circuitry, with rectangles and ovals representing enhancer elements and proteins, respectively.

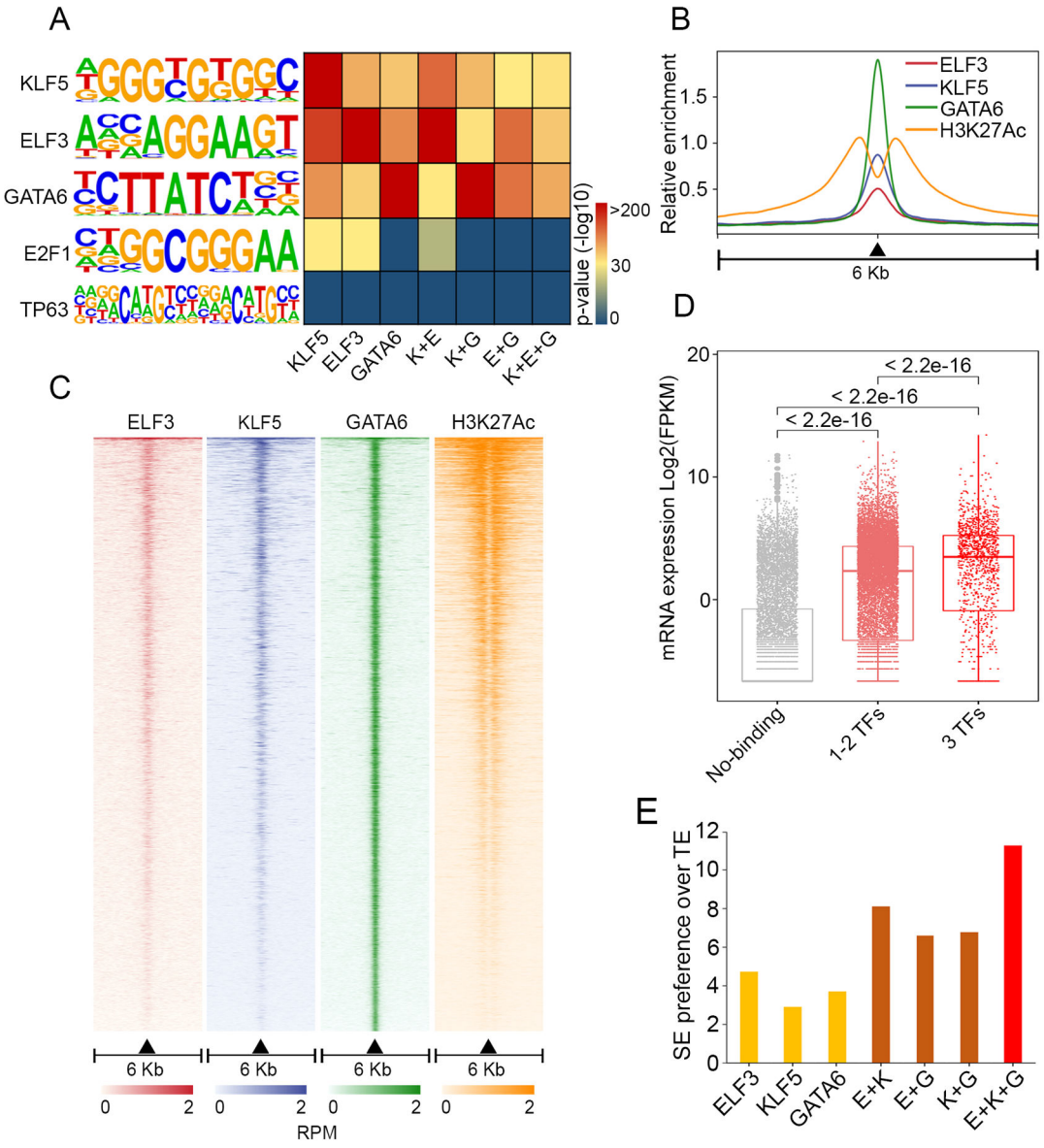

Figure 3. Master TFs orchestrate co-operatively EAC transcriptional network.

(A) Position weight matrix and heatmap showing the p values of enriched motifs in either ELF3-, KLF5-, GATA6- or co-occupied genomic regions in Eso26 cells. The enrichment of TP63 and E2F1 motifs are shown as negative controls. (B) Line plots showing the distribution of indicated ChIP-Seq signals at GATA6 peak regions (centered at the summit of GATA6 peaks). (C) Heatmap showing ChIP-Seq signals at GATA6 peak regions (+/− 3Kb of peak center), rank ordered by intensity of GATA6 peaks based on reads per million mapped reads (RPM). Lines, peaks; color scale of peak intensity is show at the bottom. (D) Box plot of mRNA levels of genes associated with each group of peaks in Eso26 cells. (E) Fold ratio of the percentage of super-enhancers (SE) over typical-enhancers (TE) bound by individual master TFs either alone or together.

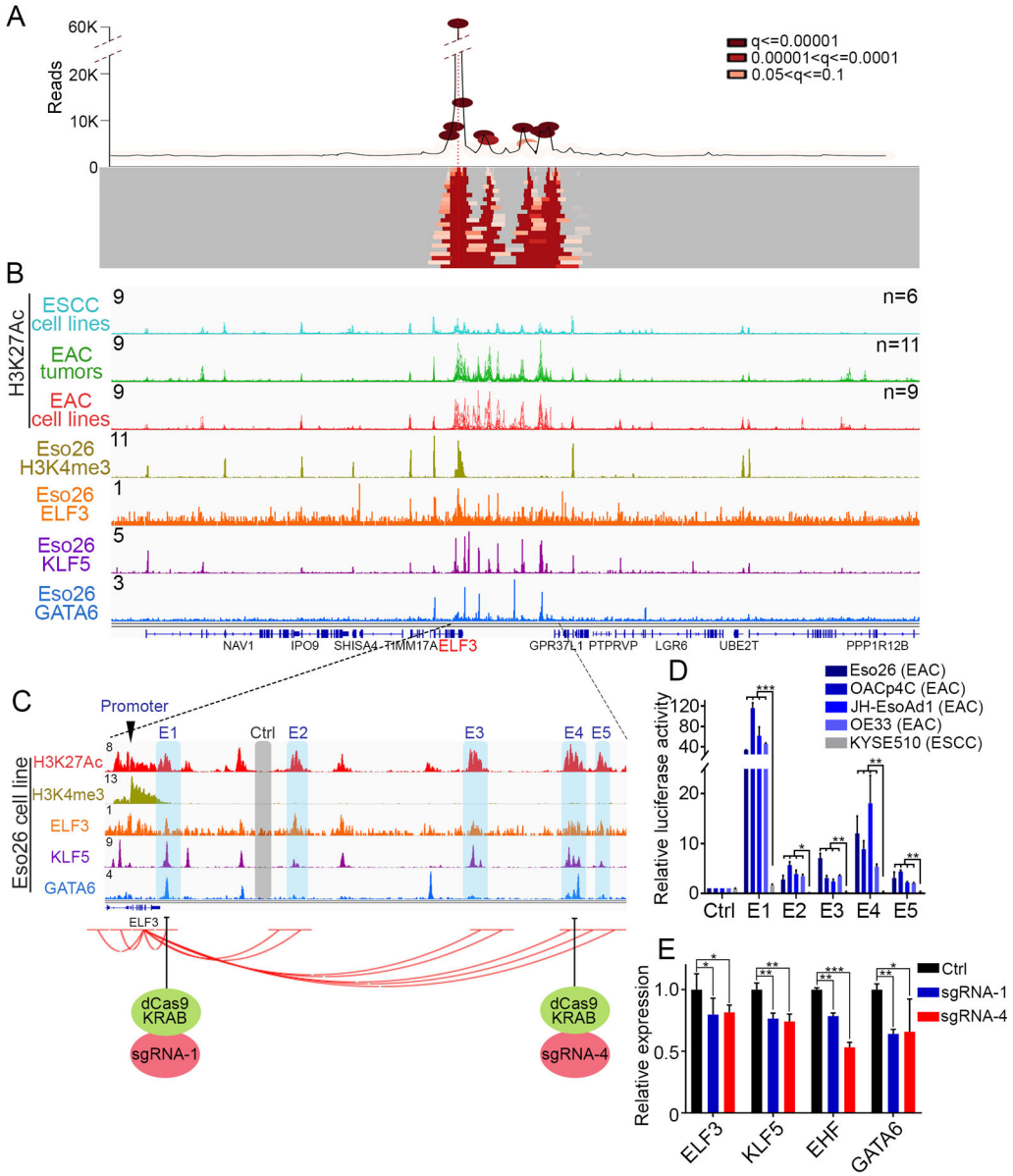

Figure 4. Master TFs co-operatively activate the super-enhancer of ELF3.

(A) 4C assay showing the long-range interactions anchored on ELF3 promoter in Eso26 cells. Deeper red color indicates higher interaction frequency. (B) ChIP-Seq profiles for H3K27Ac (in different groups of samples) and master TFs at ELF3 super-enhancer loci. (C) Zoom in view of ChIP-Seq signals in Eso26 cells. Connecting lines showing the interactions detected by 4C. Five constituent enhancers (E1-E5) and one negative control (Ctrl) region were separately cloned into luciferase reporter vector. (D) Enhancer activity measured by luciferase reporter assays in indicated EAC cells and KYSE510 cells. Mean ± s.d. are shown, n = 2. *, P<0.05; **, P<0.01; ***, P<0.001. (E) Eso26 cells expressing dCas9/KRAB vector with sgRNAs targeting E1 and E4 or control vector were subject to qRT-PCR to quantify the mRNA expression of master TFs.

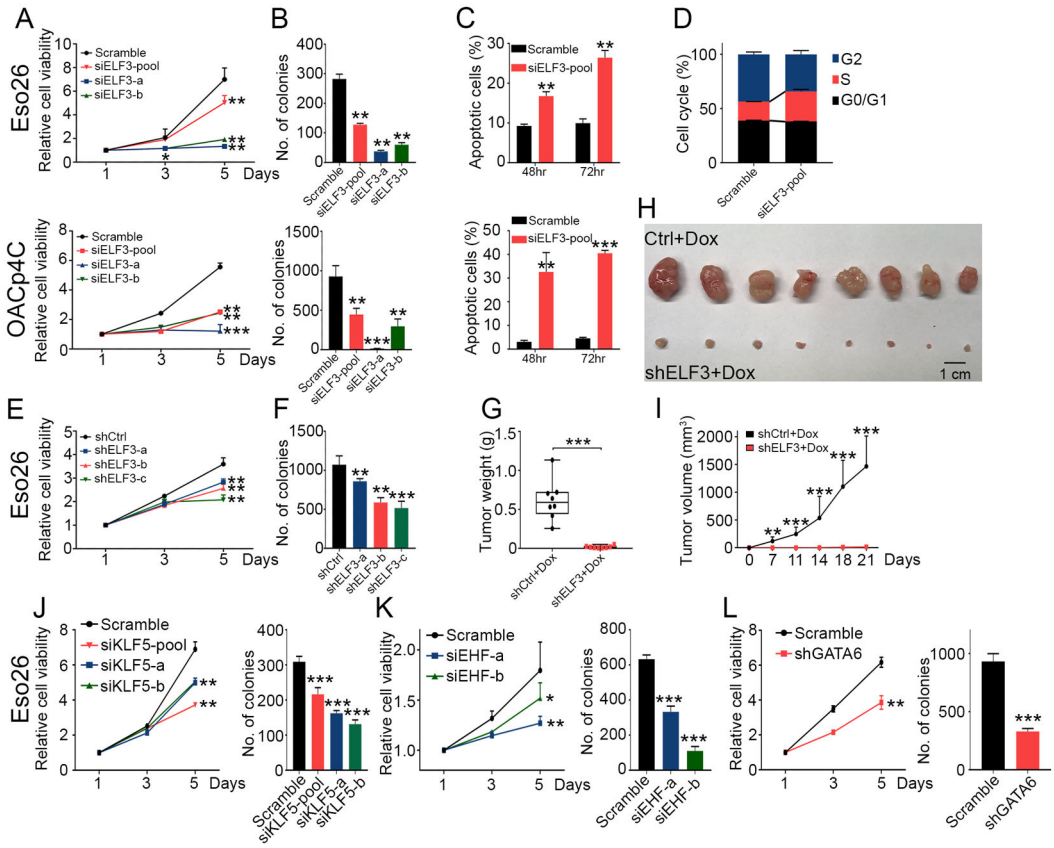

Figure 5. Master TFs have strong pro-EAC functions.

(A) Knockdown of ELF3 by individual siRNAs decreased cell proliferation and colony growth (B), and increased cell apoptosis (C) and S-phase arrest (D) in different EAC cell lines. (E) Silencing of ELF3 by inducible shRNAs in Eso26 cells decreased cell proliferation, colony growth (F) as well as xenograft growth in vivo (G-I). (G) weights, (H) images and (I) growth curves of resected tumors from both groups. (J-L) Knockdown of other three master TFs by individual siRNAs decreased cell proliferation and colony growth. Mean ± s.d. are shown, n = 3. *, P<0.05; **, P<0.01; ***, P<0.001.

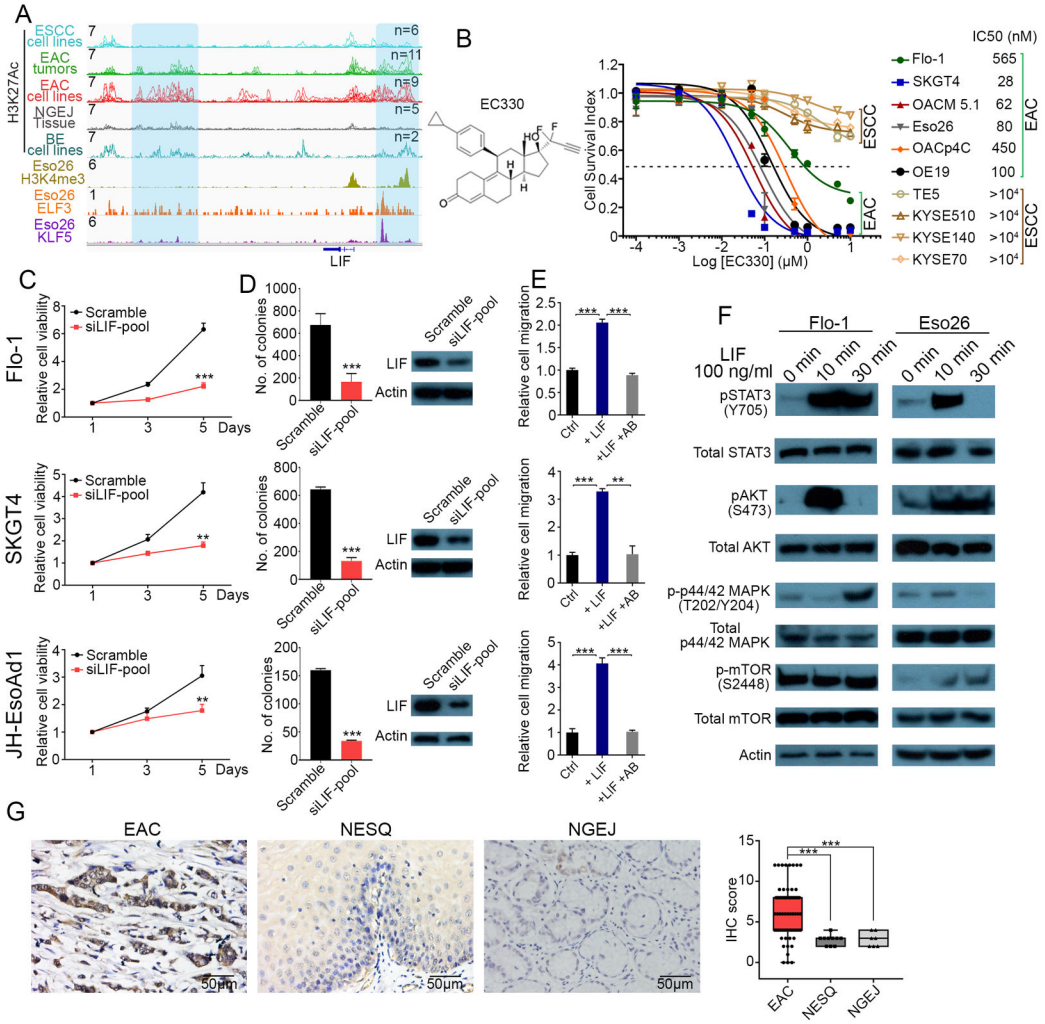

Figure 6. Up-regulated by master TFs via super-enhancers, LIF promotes EAC growth and migration.

(A) IGV plots of ChIP-Seq showing EAC-specific LIF super-enhancer which was co-occupied by master TFs. (B) Cell viability assay testing EC330, a LIF inhibitor, in EAC and ESCC cell lines. IC50 values are shown in the right panel. (C) Silencing of LIF with siRNA decreased different EAC cell proliferation and (D) colony growth. (E) LIF stimulated EAC cell migration, which was neutralized by an anti-LIF antibody. (F) Western Blotting showing that LIF stimulated STAT3 and AKT phosphorylation in EAC cell lines. Mean ± s.d. are shown, n = 3. *, P<0.05; **, P<0.01; ***, P<0.001. (G) IHC staining of LIF in EAC (n=35), non-malignant esophagus squamous mucosa (NESQ, n=10) and NGEJ samples (n=7).