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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

SOX15 Governs Transcription in Human Stratified Epithelia and a Subset of Esophageal Adenocarcinomas

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SUMMARY

This study identifies SOX15 as a direct transcriptional regulator of a substantial fraction of cell type-specific genes in stratified epithelial cells. SOX15 expression is attenuated in intestinal metaplasia (Barrett's esophagus) but is active in many esophageal adenocarcinomas.

BACKGROUND & AIMS: Intestinal metaplasia (Barrett's esophagus, BE) is the principal risk factor for esophageal adenocarcinoma (EAC). Study of the basis for BE has centered on intestinal factors, but loss of esophageal identity likely also reflects the absence of key squamous-cell factors. As few determinants of stratified epithelial cell-specific gene expression have been characterized, identifying the necessary transcription factors is important.

METHODS: We tested regional expression of mRNAs for all putative DNA-binding proteins in the mouse digestive tract and verified the esophagus-specific factors in human tissues and cell lines. Integration of diverse data defined a human squamous esophagus-specific transcriptome. We used chromatin immunoprecipitation with high-throughput sequencing (ChIP-seq) to locate transcription factor binding sites, computational approaches to profile the transcripts in cancer data sets, and immunohistochemistry to reveal protein expression.

RESULTS: The transcription factor Sex-determining region Y-box 15 (*SOX15*) is restricted to esophageal and other murine and human stratified epithelia. *SOX15* mRNA levels are attenuated in BE, and its depletion in human esophageal cells reduces esophageal transcripts significantly and specifically. SOX15 binding is highly enriched near esophagus-expressed genes, indicating direct transcriptional control. *SOX15* and hundreds of genes coexpressed in squamous cells are reactivated in up to 30% of EAC specimens. Genes normally confined to the esophagus or intestine appear in different cells within the same malignant glands.

CONCLUSIONS: These data identify a novel transcriptional regulator of stratified epithelial cells and a subtype of EAC with bi-lineage gene expression. Broad activation of squamous-cell genes may shed light on whether EACs arise in the native stratified epithelium or in ectopic columnar cells. *(Cell Mol Gastroenterol Hepatol 2015;1:598–609; http://dx.doi.org/10.1016/j.jcmgh.2015.07.009)*

Keywords: Barrett's Esophagus; Esophageal Gene Regulation; Esophageal Transcriptome; SOX15 Cistrome.

Intestinal metaplasia of the esophagus (Barrett's esophagus, BE) is a common, chronic condition in which an epithelium containing intestinal goblet and other columnar cells replaces the native stratified squamous mucosa.[1] BE results from chronic acid and bile reflux. Over time, the metaplastic tissue may become dysplastic, and it progresses to invasive cancer in three to five cases per 1000 person-years.[2] Esophageal adenocarcinoma (EAC) arises principally in the setting of BE, and the incidence of this cancer in the West increased about eightfold between 1970 and 2010, with about 18,000 new U.S. cases and 15,000 deaths expected in 2015 (http://seer.cancer.gov).

Investigation into the mechanisms of BE has centered largely on determinants of intestinal identity,[3] particularly the intestine-restricted transcription factors (TFs) Caudal type homeobox 1 (CDX1) and CDX2, which specify the embryonic intestine.[4] Forced expression of CDX2 or CDX1 in the mouse stomach induces ectopic intestinal differentiation,[5,6] and both factors are implicated in activating intestinal genes in BE,[7,8] though forced CDX2 expression in the

Abbreviations used in this paper: **BE, Barrett's esophagus; CDX1/2, caudal type homeobox 1/2; ChIP, chromatin immunoprecipitation; ChIP-seq, chromatin immunoprecipitation with high-throughput sequencing; EAC, esophageal adenocarcinoma; G-E, gastroesophageal; KRT5, keratin 5, type II; KRT6A, keratin 6A, type II; PAX9, paired box 9; PBS, phosphate-buffered saline; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; shRNA, small hairpin RNA; SIM2, single-minded family bHLH transcription factor 2; SOX2, 15, sex-determining region Y-box 2, -box 15; TCGA, The Cancer Genome Atlas; TF, transcription factor; TP63, tumor protein P63; TRIM29, tripartite motif containing 29.**

Most current article

2352-345X
http://dx.doi.org/10.1016/j.jcmgh.2015.07.009

mouse esophagus does not induce BE per se.[9] Loss of esophagus-specific transcripts and of stratified squamous morphology probably reflects parallel loss of transcriptional determinants of the native epithelium, which are largely unknown. Tumor protein P63 (TP63) regulates differentiation of all stratified epithelia, such as those in the esophagus and skin,[10,11] acting in part through another transcription factor, basonuclin 1 (BNC1).[12] Sex-determining region Y-box 2 (SOX2) controls esophageal differentiation in embryos[13] and growth of adult progenitor cells,[14,15] an activity in which Kruppel-like factor 4 (KLF4) and KLF5 also may participate.[16] Forkhead box A2 (FOXA2) is expressed in embryonic but not in adult esophageal cells.[17] We sought to identify other tissue-restricted TFs that might control the characteristic stratified epithelium.

Among all putative DNA-binding proteins, we searched first for those with esophagus-restricted expression among digestive epithelia and then for factors with attenuated expression in BE. We identified sex-determining region Y-box 15 (SOX15) as such a TF and we show that it directly controls transcription of a large fraction of human esophagus-expressed genes. *SOX15* is absent from most EACs, but up to 30% of cases retain expression of *SOX15* and its target genes, coexpressing representative intestinal and squamous-specific genes within the same tissue. Together, these data identify a novel regulator of stratified epithelial genes and a subtype of EAC with bi-lineage gene expression.

Materials and Methods

Tissue Preparation and Transcription Factor Expression Screen

We isolated epithelial sheets from the esophagus, gastric corpus-antrum, and duodenum of 1-month old CD1 and C57BL/6 mice. Before peeling the mucosa using fine forceps, the esophagus was treated with 0.1% collagenase-dispase (cat. no. 11097113001; Roche Applied Science, Indianapolis, IN) in phosphate-buffered saline (PBS) for 15 minutes at 37°C, whereas stomach and duodenum were incubated in 1 mM ethylenediaminetetraacetic acid (EDTA) in PBS at 37°C. To determine the relative transcript levels (Figure 1*A–C*), we used quantitative reverse-transcription

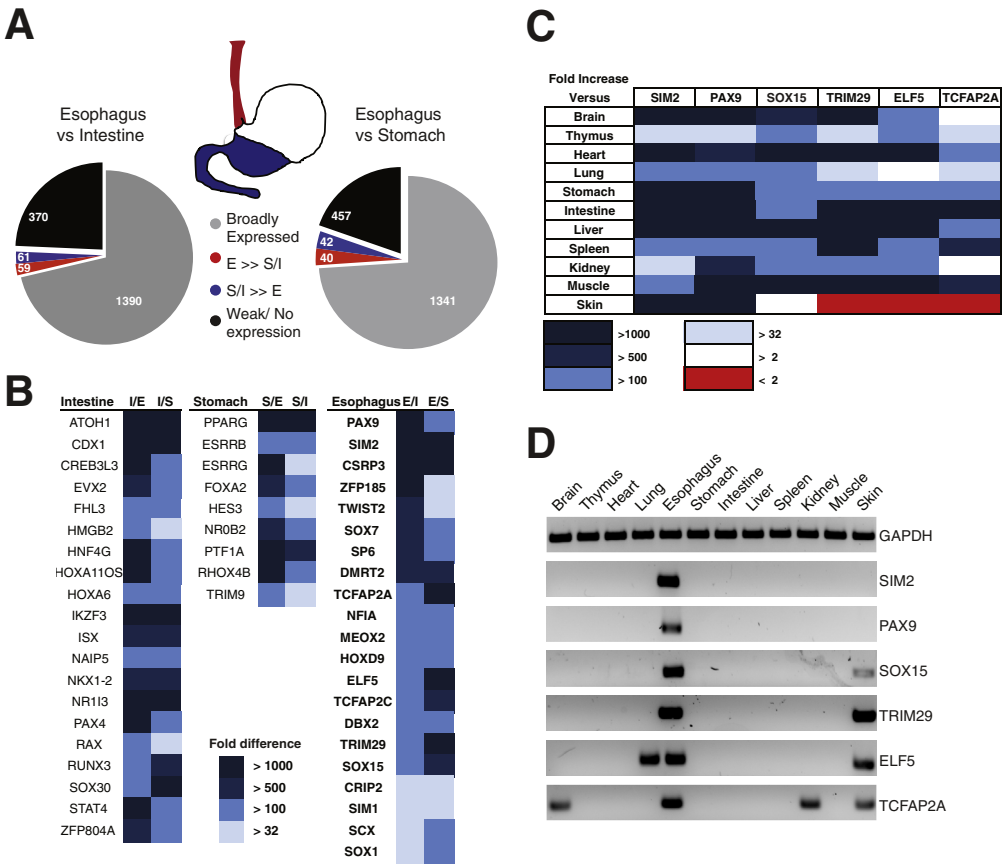
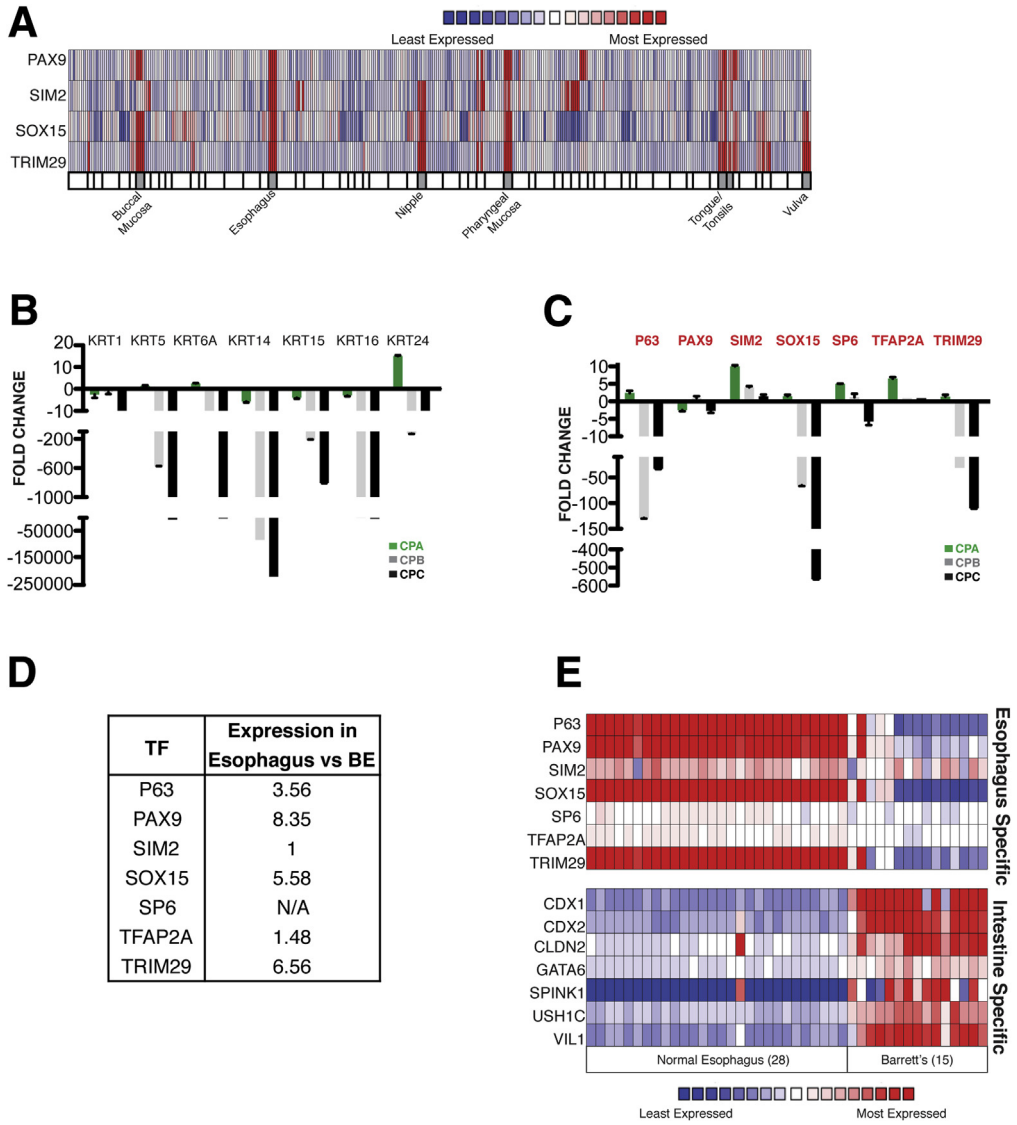


Figure 1. Differential transcription factor (TF) expression in the normal mouse gut and other tissues. (*A*) Distribution of all TFs in wild-type mouse digestive epithelia, as revealed in a quantitative reverse-transcription polymerase chain reaction (qRT-PCR) screen. Expression of 1880 TF mRNAs was assessed in epithelial cell isolates from adult CD1 mouse esophagus (*red*), stomach and intestine (*blue*). (*B*) TFs restricted to intestinal (I), stomach (S) or esophageal (E) epithelium, with the fold-excess over other tissues represented in shades of blue. (*C*) Relative expression of *Sim2*, *Pax9*, *Sox15*, *Trim29*, *Elf5*, and *Tcfap2a* mRNAs in mouse tissues. The fold-excess values are represented in shades of color as indicated in the key. (*D*) Products of qRT-PCR for the six most highly esophagus-specific TF mRNAs in 12 adult mouse organs, showing selective expression in the esophagus and of some factors in the skin.



TF	Expression in Esophagus vs BE
P63	3.56
PAX9	8.35
SIM2	1
SOX15	5.58
SP6	N/A
TFAP2A	1.48
TRIM29	6.56

Figure 2. Differential transcription factor (TF) expression in normal and metaplastic human esophagus. (*A*) Expression profiles of *PAX9*, *SIM2*, *SOX15*, and *TRIM29* in 65 human organs. Data from necropsies,[21] analyzed using Oncomine tools,[31] show selective expression in the esophagus and other stratified epithelia such as the oropharyngeal mucosa and skin derivatives. (*B*) Relative expression of esophagus-active keratin genes in the human Barrett's esophagus (BE) cell line series (CP-A, CP-B, and CP-C) with increasing dysplasia. Results of quantitative reverse-transcription polymerase chain reaction analysis are represented with respect to transcript levels in the immortalized human esophageal cell line EPC2-hTert.[20] (*C*) Relative expression of esophagus-specific TF mRNAs in human BE cell lines CP-A, CP-B and CP-C, expressed in relation to levels in EPC2-hTert cells. (*D*) Fold-enriched expression of esophagus-specific TF mRNAs in fresh human esophageal epithelial biopsy samples[8] relative to areas of BE in the same patients. (*E*) Expression of esophagus-specific TFs and intestine-specific genes in normal human esophagus and BE resection specimens. Data used[22] were analyzed using Oncomine tools.

polymerase chain reaction (qRT-PCR) and a library containing oligonucleotide primers specific to 1880 known and putative TFs.[18] Tissue-specific TFs were identified using the comparative C_T method.[19] To further determine the tissue specificity (Figure 1*D*), other whole organs were harvested from adult C57BL/6 mice.

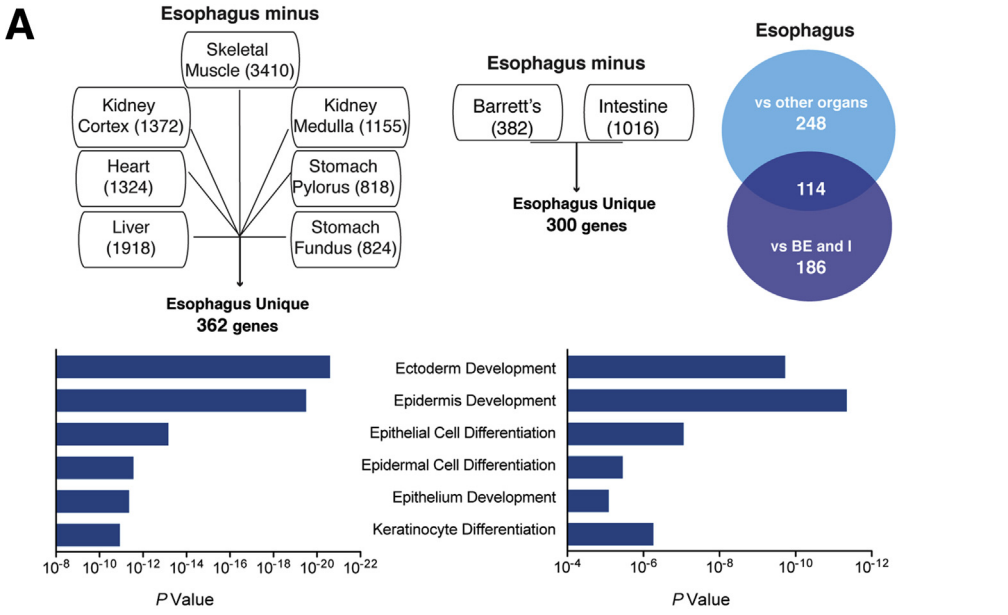
Cell Lines

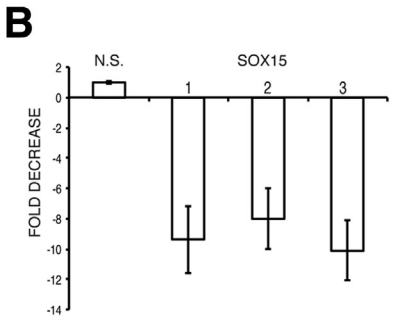
We cultured CP-A (KR-42421), CP-B (CP-52731), and CP-C (CP-94251) cells (American Type Culture Collection, Manassas, VA) in MCDB-153 medium (cat. no. M7403; Sigma-Aldrich, St. Louis, MO) supplemented with 0.4 μg/mL hydrocortisone, 20 ng/mL recombinant human epidermal growth factor (cat. no. E9644; Sigma-Aldrich), 8.4 μg/L cholera toxin (cat. no. H0135; Sigma-Aldrich), 20 mg/L adenine (cat. no. A2786; Sigma-Aldrich), 140 μg/mL bovine pituitary extract (cat. no. P1476; Sigma-Aldrich), ITS supplement (cat. no. I1884; Sigma-Aldrich) (final concentrations: 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite), 4 mM glutamine, and 5% fetal bovine serum. EPC2-hTert cells[20] were cultured in Keratinocyte-

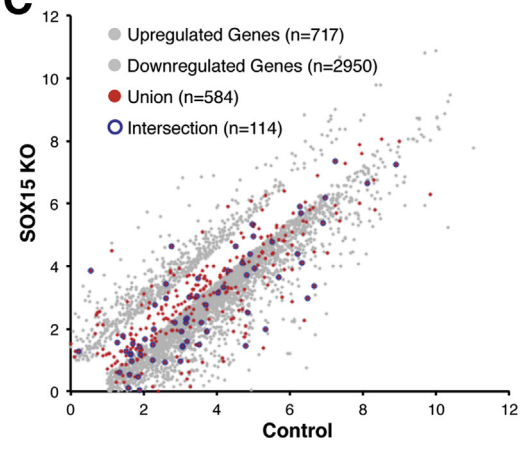
SFM medium (GIBCO/Life Technologies, Grand Island, NY) supplemented with bovine pituitary extract and recombinant human epidermal growth factor (GIBCO). Soybean trypsin inhibitor (Sigma-Aldrich) was used to quench trypsin activity during cell passage.

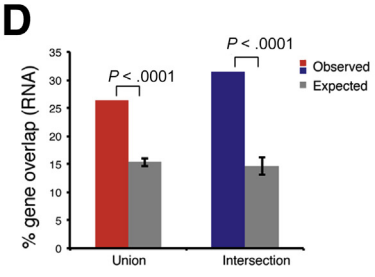
Gene Analyses

Figures 2*A*, *D*, and *E* and selected other figures show analyses of relative mRNA expression levels from published studies of 65 adult human tissues,[21] of human esophageal biopsy specimens,[8] and of human normal esophagus, BE,









	P Value
GO:0008544~epidermis development	3.09E-08
GO:0007398~ectoderm development	7.35E-08
GO:0030855~epithelial cell differentiation	1.25E-04
GO:0030216~keratinocyte differentiation	1.95E-04

and EAC samples.[22] The data were reanalyzed with respect to *SOX15* using Oncomine tools (Compendia Bioscience, Ann Arbor, MI; www.oncomine.com), considering all samples in each data set. Genes significantly associated with *SOX15* were ranked on the basis of correlation values. Enriched Gene Ontology terms were determined using DAVID tools (http://david.abcc.ncifcrf.gov/).

We examined processed RNA-seq data from a Cancer Genome Atlas (TCGA) study on stomach cancer,[23] first isolating 30 CIN^+ tumors arising at the gastroesophageal (G-E) junction or gastric cardia for unsupervised clustering (Supplementary Figure 1). To this group we applied hierarchical clustering (using hclust from the R package; http://cran.r-project.org) on the 1000 most variable transcripts normalized according to expression *z*-scores, followed by a second hierarchic clustering on the set of 317 genes coexpressed with *SOX15*. To assess the specificity of *SOX15* overexpression in tumors of the gastric cardia, we compared with RNA-seq data from TCGA studies on colon[24] and distal gastric adenocarcinomas.[23]

Experimental RNA Analyses

Total RNA was isolated using TRIzol (Invitrogen/Life Technologies, Carlsbad, CA), treated with the RNeasy Mini Kit (Qiagen, Valencia, CA), and DNA was digested using Turbo DNA-Free (Ambion/Life Technologies, Austin, TX). For qRT-PCR analysis (Figure 1*A*–*C*, Figure 2*B* and *C*, etc. 1 μg of total RNA was reverse-transcribed with Superscript III First Strand Synthesis System (Invitrogen), and cDNA was amplified using SYBRGreen PCR Master Mix (Applied Biosystems, Foster City, CA). RNA-seq libraries (the full data set is deposited in the Gene Expression Omnibus with accession number GSE62909) were prepared from 300 ng of total RNA using TruSeq RNA Sample Preparation kits (Illumina, San Diego, CA), and 75-base pair (bp) single-end sequences were obtained on a NextSeq 500 instrument (Illumina). The reads were aligned to human genome build Hg19 using TopHat v2.0.6 (https://ccb.jhu.edu/software/tophat/index.shtml). Expression levels of transcripts in duplicate samples were calculated as fragments per kb per 10^6 mapped reads (FPKM) using Cufflinks v2.0.2 (http://cole-trapnell-lab.github.io/cufflinks/), and differential expression was determined using CuffDiff (http://cole-trapnell-lab.github.io/cufflinks/cuffdiff/).[25] Chi-square tests with 1 degree of freedom and two-tailed *P* values were used to assess significance. Log_2 (FPKM + 1) values for control and *SOX15*-depleted samples were plotted to display differential expression.

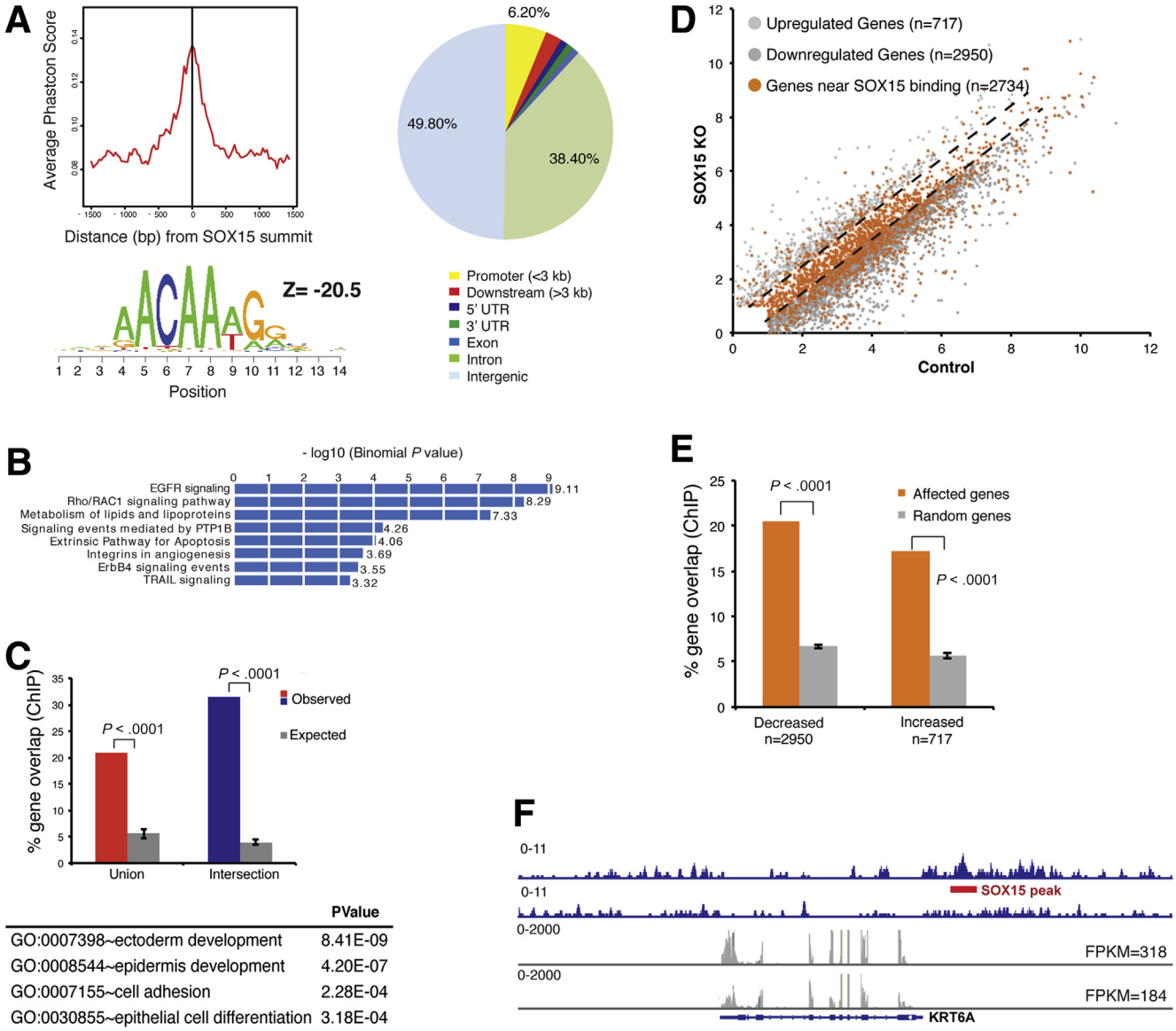
Depletion of SOX15 and Expression of Biotin-Tagged SOX15

The cells were infected with lentiviruses generated from the pLKO.1 vector (Open Biosystems/GE Dharmacon, Huntsville, AL) carrying either a *SOX15*-targeting small hairpin RNA (shRNA) (TGCCTGGCAGCTATGGCTCTT) or a control, nonspecific shRNA that does not complement any human gene and is not toxic to cultured human cells (CCTAAGGTTAAGTCGCCCTCG). Human *SOX15* cDNA was cloned into the pUltra vector (cat. no. 24129; Addgene, Cambridge, MA) together with cassettes for the T2A sequence, biotin, and BirA-V5 (gift of Ben Ebert, Brigham & Women's Hospital, Boston, MA).

Chromatin Immunoprecipitation and Chromatin Immunoprecipitation With High-Throughput Sequencing

Cells were cross-linked with 2 mM disuccinimidyl glutarate (catalog no. 20593; Pierce Biotechnology, Rockford, IL) in PBS for 45 minutes, followed by 10 minutes with 1% formaldehyde (Pierce Biotechnology) in PBS at room temperature. Chromatin immunoprecipitation (ChIP) and chromatin immunoprecipitation with high-throughput sequencing (ChIP-seq) were performed as described previously elsewhere[26] using a 30-μL slurry of streptavidin-conjugated magnetic beads (cat. no. 65601; Invitrogen). We used Cistrome tools (www.cistrome.org) to identify and annotate TF binding sites, generate wiggle files and conservation plots, identify enriched sequence motifs and linked genes, and compare data across ChIP-seq libraries. Wiggle traces were projected on the Integrative Genome Viewer (www.broadinstitute.org/igv/).[27] Functions of genes within 50 kb of SOX15 occupancy were determined using GREAT (http://omictools.com/great-s1664.html).[28] ChIP-seq data are deposited in the Gene Expression Omnibus (GEO) database with accession number GSE62909 (www.ncbi.nlm.nih.gov/geo/).

Figure 3. (***See previous page***). **Impact of SOX15 depletion on esophageal gene expression.** (*A*) Delineation of the human esophageal transcriptome. The mRNAs expressed in human esophageal necropsy specimens (*left*) were compared against transcripts from 7 other postmortem organs,[21] and mRNAs present in fresh esophageal biopsy specimens (*right*) were compared against transcripts from fresh Barrett's esophagus (BE) and intestinal biopsies.[8] Numbers in each box represent squamous esophagus-specific genes relative to that tissue. We identified 362 and 300 esophagus-specific genes, respectively, with a significant 114-gene overlap ($P < .0001$, chi-square test). The top Gene Ontology (GO) terms in each case are highly related to stratified epithelia. (*B*) *SOX15* mRNA depletion in 3 representative experiments in which CPA cells were infected with lentiviruses carrying *SOX15*-specific or a nonspecific (NS) 21-bp shRNAs. Knockdown efficiency, assessed by quantitative reverse-transcription polymerase chain reaction 72 hours after infection, was >8- to 10-fold in every experiment. (*C*) Results of duplicate RNA-seq analysis of genes differentially expressed in CP-A cells treated with *SOX15*-specific (*y*-axis) or control, nonspecific (*x*-axis) shRNAs. Grey dots mark differential expression (log_2 >1.5-fold, $q < .05$); genes present in the union (548 genes) or intersection (114) sets of esophagus-specific genes are represented by red and blue dots, respectively. (*D*) Fraction of esophagus-specific transcripts reduced upon SOX15 depletion (*red,* 548 union-set genes; *blue,* 114 intersection-set genes) compared with five random sets of equal numbers of genes expressed in CP-A cells (*grey bars*). The table lists GO terms enriched among SOX15-dependent genes.



	PValue
GO:0007398~ectoderm development	8.41E-09
GO:0008544~epidermis development	4.20E-07
GO:0007155~cell adhesion	2.28E-04
GO:0030855~epithelial cell differentiation	3.18E-04

Figure 4. Genome-wide SOX15 occupancy and gene dependence in human esophageal cells. (*A*) Summary of chromatin immunoprecipitation (ChIP) analysis for biotinylated SOX15 in CP-A cells, showing high sequence conservation and significant enrichment of a canonical SOX recognition motif ACAA(A/T)G among 4864 identified binding sites. SOX15 mainly binds DNA far from promoters. (*B*) Gene Ontology (GO) terms enriched among the two nearest genes within 50 kb of SOX15-binding sites, determined using GREAT.[28] (*C*) Percentages of esophagus-specific genes (as determined in Figure 2*A*, *red*: union; *blue*: intersection set) that bind SOX15 within 50 kb of the transcription start site (TSS), compared with five random gene sets of equivalent size (*grey bars, P* < .0001). The table lists GO terms enriched among genes from the esophagus transcriptome that lie within 50 kb of SOX15 binding sites. (*D*) SOX15 binding (*orange dots*) within 50 kb of genes expressed in SOX15-depleted and control CP-A cells (*grey dots, q* < .05, as in Figure 2*B*). Dashed lines demarcate genes unaffected by SOX15 loss. (*E*) Genes reduced or increased in SOX15-depleted cells are significantly enriched for nearby SOX15 binding. Together with the proportions of orange dots in *D*, the data imply direct SOX15 activation of many, and direct repression of fewer genes. (*F*) Integrated Genome Viewer representation of esophageal gene *KRT6A*, showing SOX15 binding at the locus (*top rows, blue,* ChIP-seq tags) and reduced expression in *SOX15*-depleted CP-A cells (*bottom rows, grey,* RNA-seq tags). Numbers represent the height of the *y*-axis.

Immunohistochemistry

We baked 4-μm-thick tissue paraffin sections overnight at 37°C; they then were deparaffinized in xylenes, rehydrated, and peroxidase activity was blocked with 1.5% H_2O_2 in methanol for 10 minutes. Slides were treated with 0.01 M citrate buffer, pH 6.0, in a pressure cooker at 120°C for 30 minutes for antigen retrieval, then transferred to Tris-buffered saline. Sections were first incubated with mouse CDX2 Ab (clone CDX2-88, Biogenex mu392A-uc, 1:200) for 40 minutes, followed by Dako Envision+ Mouse (Dako K4007; Dako, Carpinteria, CA) secondary Ab for 30 minutes, and developed with 3,3′-diaminobenzidine (Dako). Sections were

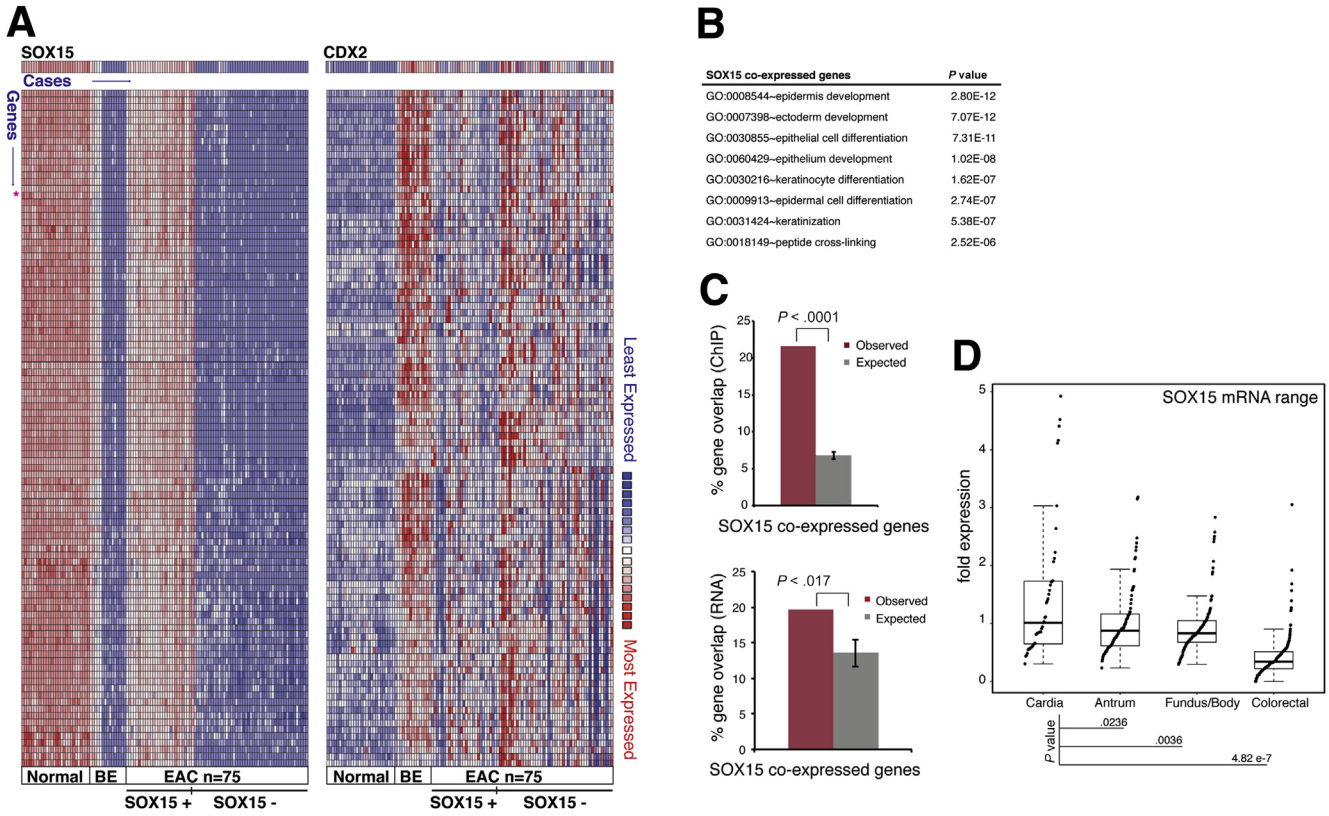


Figure 5. SOX15 expression in esophageal adenocarcinomas (EACs). (*A*) Gene coexpression profiles for *SOX15* (left) and *CDX2* (*right*) in a large collection of normal, Barrett's esophagus (BE), and EAC epithelium.[22] We found that 317 transcripts correlated strongly ($r > 0.81$) with *SOX15* mRNA levels in normal esophagus and in approximately one-third of 75 EACs in this series. The 100 most highly correlated genes are shown. (*B*) Gene Ontology (GO) term enrichment among these 317 *SOX15*-coexpressed genes. (*C*) *Top*: Fraction of *SOX15* coexpressed genes showing SOX15 occupancy (observed) within 50 kb compared with the fraction expected for appropriate random gene sets of equal size. *Bottom*: Fraction of *SOX15* coexpressed genes affected by SOX15 depletion (observed) compared with the fraction expected among random gene sets of equal size. (*D*) Ranges of *SOX15* mRNA expression extracted from RNA-seq data on the Cancer Genome Atlas collection of cancers of the gastric cardia, fundus/body, and antrum,[23] or colon and rectum.[24] Statistical significance of the differences was determined by *t*-test.

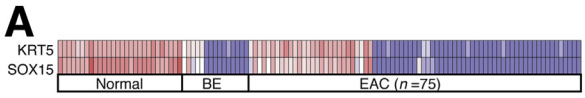
then incubated with mouse KRT5 Ab (clone XM26, Leica NCL-L-CK, 1:500) for 40 minutes, followed by PowerVision AP mouse (catalog no. PV6110; Leica Biosystems, Buffalo Grove, IL) secondary Ab for 30 minutes, developed with Permanent Red, and counterstained with Mayer's hematoxylin. To stain resection specimens that carried areas of BE, slides were treated with the same mouse KRT5 Ab, followed by Dako Envision+ Mouse (Dako K4007) secondary Ab for 30 minutes, and developed with 3,3′-diaminobenzidine (Dako).

Results

Identification of Transcription Factors That Are Specific to the Esophageal Epithelium and Attenuated in Barrett's Esophagus

To identify candidate regulators of esophageal squamous identity, we first examined epithelia isolated from different regions of the mouse alimentary tract—esophagus, glandular stomach, and intestine (duodenum)—with a goal to identify TF mRNAs expressed selectively in the stratified esophageal epithelium (Figure 1*A*). Among 1880 known and putative DNA-binding proteins, those showing ≥32-fold higher expression in the intestinal mucosa included the known intestinal factors *Atoh1*, *Cdx1*, *Creb3l3*, *Hnf4g*, and *Isx*,[29] underscoring the fidelity of the experimental approach (Figure 1*B*). Forty factors and 59 TF genes showed considerably higher expression in esophageal cells, compared with the gastric corpus and the intestine, respectively (Figure 1*A*), and 21 TFs were common to the two esophagus-specific groups (Figure 1*B*).

To exclude variability among mouse strains and to assess specificity relative to nondigestive organs, we measured expression of these 21 mRNAs in nine diverse tissues from C57BL/6 mice, including the skin. Six TFs gave consistent evidence of high tissue specificity (Figure 1*C* and *D*). *Sim2* (single-minded family bHLH transcription factor 2) and *Pax9* (paired box 9) showed the greatest specificity, followed by *Sox15* and *Trim29* (tripartite motif containing 29), which showed some expression in murine skin. Additional data from 65 adult human tissues[21] revealed robust expression of each of these four TF mRNAs in the esophagus, with varying levels in other stratified squamous



B

Total EAC Cases	KRT5 positive %	CDX2 positive %	Both expressed %
99	19	83	18

EAC cases with positive KRT5 staining	IHC score KRT5
4	+++
7	++
8	+
EAC cases with positive CDX2 staining	**IHC score CDX2**
49	++++
16	+++
15	++
3	+

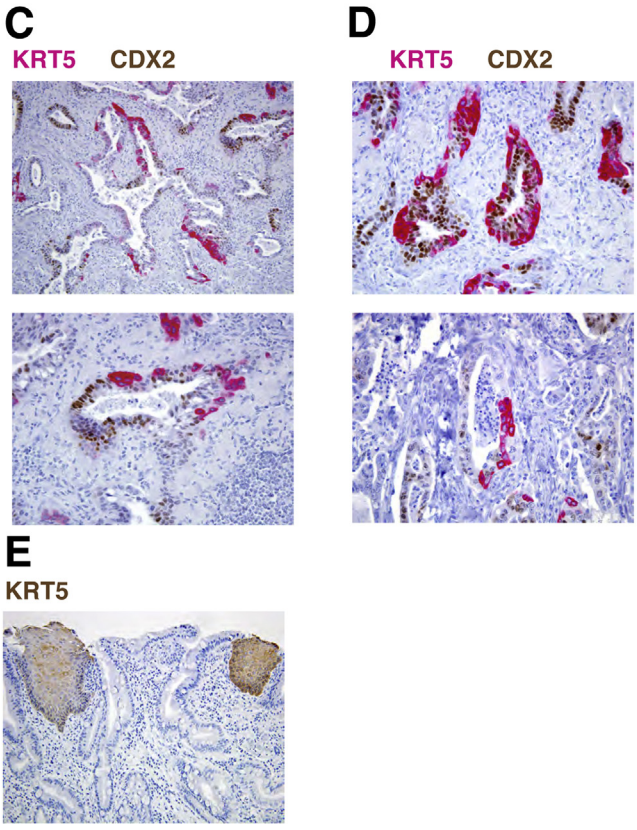


Figure 6. Bi-lineage gene expression in a subset of esophageal adenocarcinomas (EACs). (*A–B*) High correlation ($r = 0.97$) of *SOX15* and *KRT5* mRNAs in normal esophagus, Barrett's esophagus (BE), and EAC, validating *KRT5* as a proxy for *SOX15* and other stratified epithelium-specific genes. (*B*) Table of IHC results for KRT5 and CDX2 in 99 cases of EAC. (*C–D*) Representative immunohistochemistry for KRT5 (*red*, a surrogate marker for SOX15 and other squamous-specific gene products) and CDX2 (*brown*, a representative intestine-specific marker) in two separate cases (*C* and *D*) of human EAC. High KRT5 expression is evident, with almost mutually exclusive distribution of KRT5 (++ to +++) and CDX2 (+++ to ++++) in the same malignant glands. Original magnifications: Top, 200×; Bottom, 400×. (*E*) Absence of KRT5 immunostaining in areas of BE. Adjoining areas of normal stratified epithelium provide a positive control and contrast. IHC, immunohistochemistry.

tissues, such as the tongue, mouth, pharynx, and skin derivatives (Figure 2*A*).

To determine whether these TFs may function in the identity of stratified epithelia, we examined expression data from immortalized EPC2-hTert esophageal keratinocytes[20] and found high expression of each factor except *ELF5* (data not shown). Next we tested a series of three cell lines: CP-A, which represents nondysplastic BE, and CP-B and CP-C, which represent BE with high-grade dysplasia. This cell line series replicates disease progression[30] with reduced levels of multiple keratin mRNAs (Figure 2*B*).

We observed a concomitant decline in *SOX15* and *TRIM29* levels, matching or exceeding that of *TP63* mRNA, with little variance in the other factors (Figure 2*C*). Although these findings do not in isolation give robust information about a relation to mucosal dysplasia per se, they reveal the squamous cell specificity of *SOX15* and *TRIM29*. Furthermore, gene expression data from a collection of human esophageal biopsy specimens[8] showed significantly fewer *SOX15* and *TRIM29* mRNAs in primary BE compared with adjacent normal esophageal mucosa (Figure 2*D*).

Finally, we used Oncomine tools[31] to analyze mRNA data from an independent series of 28 frozen human normal esophagus and 15 frozen BE biopsy specimens.[22] Levels of *PAX9*, *SOX15*, and *TRIM29* were uniformly high in normal esophagus and attenuated in BE specimens (Figure 2*E*).

Together, these data identify SOX15, PAX9, and TRIM29 as conserved candidate determinants of squamous cell identity. *PAX9* levels were similar in CP-A, CP-B and CP-C cells (Figure 2*C*), and although TRIM29 has a putative DNA-binding domain, its role in transcriptional regulation is poorly defined and uncertain.[32] By contrast, SOX proteins control differentiation of diverse tissues, often in conjunction with other family members,[33] and related factors such as SOX2 and SOX7 are known to regulate aspects of esophageal organogenesis and squamous cell cancer.[13,14] We therefore concentrated on human SOX15, which shares 85% homology (100% in the DNA-binding domain) with the mouse protein. *SOX15* was previously noted as one among hundreds of genes in various expression profiling studies,[34–36] and we proceeded to investigate its functions.

SOX15 *Depletion Affects Genes Specific to Stratified Epithelium*

To test whether SOX15 might regulate genes specific to the stratified human esophageal epithelium, we needed to delineate the corresponding transcriptome. To this end, first we considered public data from adult human postmortem tissues[21] (see Figure 2*A*) and identified 362 genes that express at greater than threefold higher levels ($P < .05$) in the esophagus than in any of seven diverse tissues, including glandular stomach, from the same collection of postmortem samples (Figure 3*A*). Second, we identified 300 genes with greater than threefold higher mRNA levels ($P < .05$) in normal fresh human esophagus biopsy specimens than in adjacent areas of BE or in fresh intestinal biopsies from the same study.[8] Consistent with specific roles in stratified epithelia, both gene sets were highly enriched for functions

related to ectodermal, epidermal, and keratinocyte differentiation, and they shared 114 genes (Figure 3*A*). Accordingly, we regard the union set of 548 genes as a good representation of human esophagus-specific transcripts and the intersection set of 114 genes as an especially robust subset.

To determine whether SOX15 regulates any part of this transcriptome, we used lentiviral delivered shRNA to deplete the TF in CP-A cells. These cells express keratin and TF genes specific to stratified epithelia, including *SOX15,* at levels similar to immortalized EPC2-hTert esophageal epithelial cells (Figure 2*B* and *C*), and they tolerate lentiviral infection and drug selection. Because *SOX15* depletion retarded CP-A cell growth and survival, we harvested cells 72 hours after infection, when they appeared healthy but the *SOX15* mRNA levels were appreciably reduced (Figure 3*B*).

RNA-seq analysis showed reduced and increased levels of 2950 and 717 transcripts, respectively, compared with cells treated with a nonspecific shRNA (Figure 3*C*). In agreement with the deficit in cell growth, these genes were enriched for Gene Ontology terms related to the cell cycle (Supplementary Table 1). More importantly, genes reduced in *SOX15*-depleted cells included 26.4% of the human esophagus-specific "union" transcriptome, compared with 15.34% overlap with multiple sets of 2950 random genes expressed in CP-A cells (Figure 3*D*; $P < .0001$). Correspondence was even higher for the esophagus-specific "intersection" transcriptome, where 31.5% of genes were reduced in *SOX15*-depleted cells compared with 14.68% of random genes ($P < .0001$). SOX15-dependent genes were highly enriched for functions related to stratified epithelia (Figure 3*D*). None of the 114 genes in the esophagus-specific "intersection set," and only 23 genes in the "union set" were increased in SOX15-depleted CP-A cells, and we observed no increase in intestinal genes. Rather, the 717 increased transcripts were enriched for functions such as apoptosis and vesicular transport (Supplementary Table 1). Thus, beyond cell survival or proliferation, a substantial portion of the esophageal transcriptome depends on SOX15.

SOX15 *Directly Regulates Esophagus-Specific Genes*

Depletion of SOX15 could affect transcript levels as a consequence of its *cis*-regulatory activity or indirectly. To determine whether SOX15 might regulate dependent genes directly, we used ChIP-seq to map its cistrome. Because available antibodies performed poorly in ChIP assays, we expressed biotin-tagged SOX15 stably in CP-A cells and precipitated chromatin using streptavidin beads. The nearly 5000 high-confidence binding sites we identified by this approach showed high sequence conservation and greatest enrichment for the SOX consensus motif, which was present in >97% of sites, implying direct TF occupancy (Figure 4*A*). Similar to other tissue-specific TFs, SOX15 occupied few promoters (6.2% of all binding sites) and bound DNA predominantly in intergenic regions and introns (Figure 4*A*).

GREAT analysis[28] of the nearest flanking genes within 50 kb of SOX15 occupancy revealed enrichment of pathways known to be vital in stratified epithelia, such as epidermal growth factor and Rho/Rac signaling, and in cell survival (Figure 4*B*). Moreover, 20.9% of genes in the human esophagus-specific transcriptome and 31.5% of genes common to the two esophagus transcript sources showed at least one SOX15-binding site within 50 kb of the transcription start site, compared with about 5% of comparable numbers of random genes ($P < .0001$, Figure 4*C*).

Gene Ontology terms related to stratified epithelia were further enriched among SOX15-bound genes (Figure 4*C*). Most importantly, genes affected by SOX15 depletion in CP-A cells were highly enriched for nearby SOX15 binding, compared with random gene sets of equal size ($P < .0001$), and SOX15-bound genes reduced in SOX15-depleted cells far outnumbered genes that were increased (Figure 4*D* and *E*).

Taken together, these data indicate direct SOX15 regulation of genes specific to the stratified squamous epithelium, with a strong bias toward gene activation. Canonical esophageal genes such as *KRT6A* (keratin 6A, type II) illustrate SOX15 occupancy at putative *cis*-regulatory sites and reduced expression in SOX15-depleted cells (Figure 4*F*).

SOX15 *in Human Esophageal Adenocarcinoma*

SOX15 is expressed highly in normal human esophagus, but not in the BE cell lines CP-B and CP-C (Figure 2*C*) or in areas of intestinal metaplasia in vivo (Figure 2*D* and *E*). To our surprise, RNA expression data from a large collection of frozen primary esophagus, BE, and EAC biopsy specimens[22] revealed high *SOX15* mRNA levels in up to one-third of EACs (Figure 3*A*, left; note that all samples in this study, including EAC, were frozen biopsy specimens). Moreover, at least 317 transcripts that are strongly coexpressed with *SOX15* in the normal esophagus ($r > 0.81$; Supplementary Table 2) were also present in the same EAC specimens (Figure 5*A*, which shows the 100 genes with highest correlation), suggesting broad activation of the squamous cell transcriptional program.

Accordingly, functions related to stratified epithelia were significantly enriched among the genes coexpressed with *SOX15* (Figure 5*B*). The canonical intestinal marker *CDX2* and its coexpressed genes ($r > 0.81$) were expressed in many $SOX15^{+}$ and also in $SOX15^{-}$ specimens (Figure 5*A*, right), revealing coexpression of esophageal and intestinal genes in some cases. Moreover, 21.6% of genes coexpressed with *SOX15* in this analysis showed SOX15 binding within 50 kb in CP-A cells, compared with ~6% of random genes ($P < .0001$; Figure 5*C*, top), which implies that many of these genes are direct transcriptional targets. Indeed, the effects of SOX15 depletion were statistically significantly greater on these genes than on random sets of genes expressed in CP-A cells ($P < .017$; Figure 5*C*, bottom). These features collectively suggest direct SOX15 regulation of many esophagus-restricted genes that are silent in BE and reactivated in up to one-third of human EACs.

To exclude the possibility that EACs expressing *SOX15* were simply contaminated with normal $SOX15^{+}$ esophageal cells, we studied cases from an independent collection, the Cancer Genome Atlas (TCGA), where nonmalignant cells were meticulously minimized.[23] Cancers of the G-E junction

typically arise in a background of BE and, when associated with chromosomal instability (CIN), usually represent distal EACs. Among 30 cases of CIN+ tumors from the G-E junction or gastric cardia in the TCGA collection of gastric cancers, some samples showed robust levels of *SOX15* and of genes coexpressed with *SOX15* in normal esophageal epithelium (Supplementary Figure 1*A* and *B*). Transcripts specific to the squamous esophageal epithelium were thus again evident in a fraction of EACs. To determine whether this extent of SOX15 expression is specific to EACs, we evaluated other gastrointestinal cancers in the TCGA collection: gastric fundus, body, and antrum, and colorectal tumors. Extreme outliers for *SOX15* mRNA expression were present only among tumors of the gastric cardia (Figure 5*D*).

Finally, we examined 99 separate EACs by immunohistochemistry on resection specimens. Because several antibodies failed to detect SOX15, we used KRT5 (keratin 5, type II) as a proxy for expression of SOX15 and other stratified cell-specific products, noting nearly total concordance of *SOX15* and *KRT5* mRNA expression in the large aforementioned tissue collection[22] (Figure 6*A*; $r = 0.97$). We also stained the same samples for the intestinal marker CDX2. Nineteen cases (19%) showed cytoplasmic KRT5 expression within malignant glands, and most of these cases coexpressed nuclear CDX2 (Figure 6*B*). Levels of KRT5 were variable (Figure 6*B*) but did not correlate with tumor grade or other pathologic features such as mucin production. Importantly, KRT5 was not expressed in rare pockets of squamous differentiation but rather in bona fide glandular structures. In fact, and of particular note, cytoplasmic KRT5 and nuclear CDX2 were almost always present in different cells within the same glands (see Figures 6*C* and *D* for examples from two different cases). Coexpression of esophagus- and intestine-specific genes within individual glands reveals the malignant cells' potential to express genes from distinct cell lineages.

To corroborate the observation that mRNA levels of stratified cell genes are low in BE but elevated in many EACs (Figures 5*A* and 6*A*), we used immunohistochemistry to assess areas of BE that were present in 24 of the 99 resection specimens. KRT5 was uniformly absent from these areas, though the signal was clear in adjoining stratified epithelium (Figure 6*E*). These findings extend previous reports of absent expression of stratified epithelium-specific keratins in BE[37–39] and low-level expression of squamous cell products in EACs.[40] Our delineation of a squamous cell-restricted transcriptome (Figure 3*A*), coupled with reanalysis of published RNA expression data (Figure 5*A* and *B*) and investigation of additional cases by immunohistochemical analysis (Figure 6*B*–*D*), reveals for the first time the extent and breadth of an aberrant stratified-cell program in EACs.

Discussion

Implications for Esophageal Squamous Differentiation and Esophageal Adenocarcinoma

Insights into transcriptional control of the esophageal squamous epithelium are largely limited to the broad functions of TP63 and SOX2.[10,13] Our identification of SOX15 as a novel, conserved, and likely direct regulator of many human stratified epithelial genes extends our understanding of esophageal differentiation and pathology. The lack of overt esophageal defects in *Sox15* mutant mice[41,42] is compatible with the considerable known redundancies among SOX-family TFs.[33]

There is much debate whether BE and particularly EAC originate in the native esophageal epithelium through bona fide metaplasia or in ectopic cells that may colonize the esophagus from the gastric cardia, as in mice.[11,43] Clearly, the best way to answer the question is through lineage-tracing studies, which are possible in animals but not in humans. When lineage tracing is not feasible, cell-specific transcript patterns offer clues, and the expression of *SOX15* and other squamous epithelial genes may be informative. Consider, for example, the observation that the BE cell lines CP-A/B/C show reduced levels of *SOX15*, esophageal keratins, *TP63*, and other esophagus-specific TF genes, implying loss of an esophageal program, but some esophagus-restricted TF genes such as *SIM2* and *TFAP2A* (transcription factor activating enhancer binding protein 2α) are highly expressed in these cells (Figure 2*C*) and in BE biopsy specimens (Figure 2*D* and *E*). Moreover, >300 esophageal genes, including *SOX15*, are active in up to 30% of human EACs (Figure 3*A*), with intestinal genes such as *CDX2* often coexpressed in the same glands as esophageal genes (Figure 4*C* and *D*). These findings in BE and EAC could indicate residual squamous cell-specific transcription or fortuitous ectopic gene activity. If diseased cells are better equipped to express genes from their native transcriptional program than are genes from a heterologous cell lineage, because native genes and their *cis*-regulatory elements are inherently primed and accessible, then the first possibility may be more likely. Our findings do not of course rule out the alternative model, which will require additional, independent lines of evidence.

EAC is a particularly recalcitrant disease, with poor 5-year survival rates. Surgery and empiric cytotoxic chemotherapy anchor current treatment approaches,[44,45] and although disease heterogeneity is apparent in the clinic, the underlying determinants are unclear. We show here that one-fifth to one-third of EACs simultaneously express products specific to the esophageal squamous epithelium and columnar intestinal cells. It will be important in the future to identify the clinical and genetic correlates of these EACs showing bi-lineage gene expression and to determine whether they reflect a distinctive pathophysiology or harbor unique therapeutic vulnerabilities.

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Received December 5, 2014. Accepted July 27, 2015.

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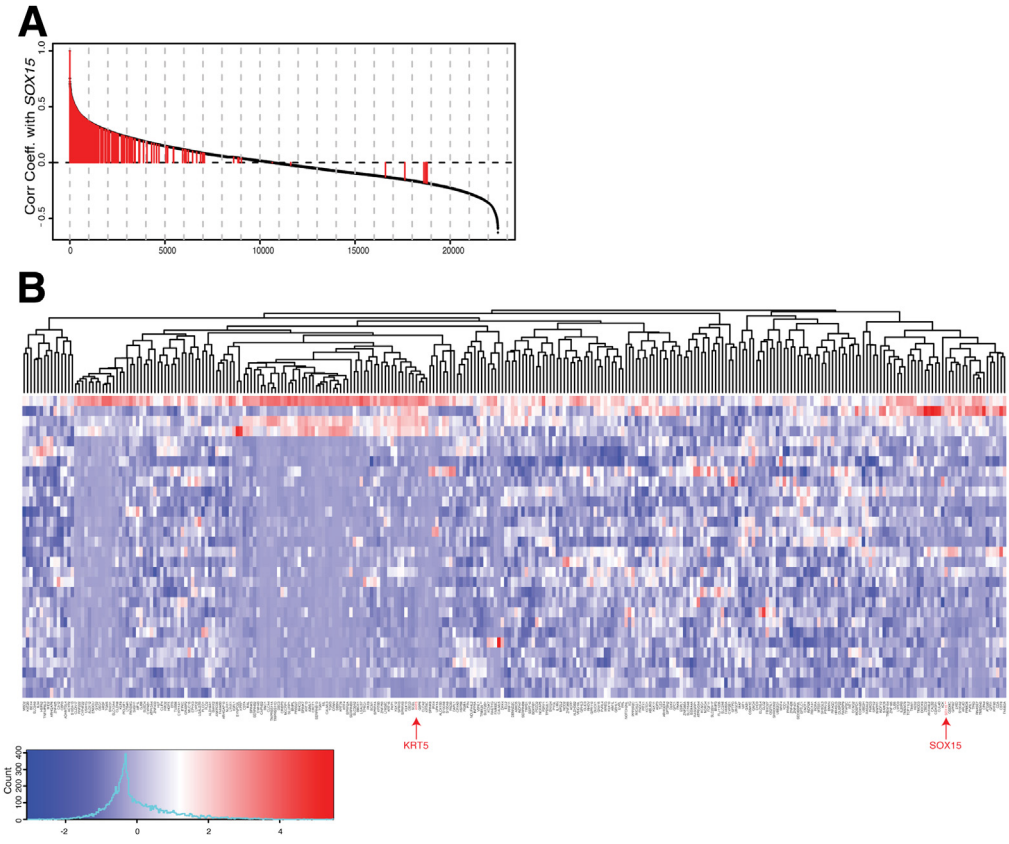
Acknowledgments
The authors thank Rishi Puram and Ben Ebert for BirA-V5 vectors and Austin Dulak and Hiroshi Nakagawa for assistance with esophageal cell culture.

Conflicts of interest
The authors disclose no conflicts.

Funding
This study was funded by the DFCI-Novartis Drug Discovery Program, generous gifts from the Lind family, and National Institutes of Health grants R01DK081113 (R.A.S), P01CA098101 (A.J.B. and A.K.R.), and P50CA127003 (Dana-Farber/Harvard).



Supplementary Figure 1. Genes coexpressed with *SOX15* in the Cancer Genome Atlas (TCGA) data set of gastric cancers. (*A*) Correlations of all mRNAs with *SOX15* levels. The 317 genes coexpressed with *SOX15* in esophageal epithelium are marked with red lines. (*B*) Expression of SOX15 coexpressed genes in 30 cases of CIN^{+} (chromosomal instability) adenocarcinomas from the gastroesophageal junction or gastric cardia.

Supplementary Table 1. Top Gene Ontology (GO) terms for transcripts altered after *SOX15* depletion in CPA cells

Term RNAseq Down	*P* Value	Term RNAseq UP	*P* Value
GO:0000278 ~ mitotic cell cycle	3.26E−20	GO:0000079 ~ regulation of cyclin-dependent protein kinase activity	4.40E−06
GO:0051301 ~ cell division	6.39E−20	GO:0016192 ~ vesicle-mediated transport	6.89E−06
GO:0000280 ~ nuclear division	1.39E−19	GO:0051726 ~ regulation of cell cycle	8.53E−06
GO:0007067 ~ mitosis	1.39E−19	GO:0009991 ~ response to extracellular stimulus	3.09E−05
GO:0022403 ~ cell cycle phase	1.48E−19	GO:0006091 ~ generation of precursor metabolites and energy	5.73E−05
GO:0000087 ~ M phase of mitotic cell cycle	1.52E−19	GO:0045767 ~ regulation of antiapoptosis	7.05E−05
GO:0022402 ~ cell cycle process	4.58E−19	GO:0031667 ~ response to nutrient levels	1.71E−04
GO:0007049 ~ cell cycle	2.49E−18	GO:0007584 ~ response to nutrient	2.63E−04
GO:0000279 ~ M phase	2.96E−18	GO:0008219 ~ cell death	5.87E−04
GO:0048285 ~ organelle fission	3.08E−18	GO:0010033 ~ response to organic substance	6.29E−04
GO:0007059 ~ chromosome segregation	1.93E−10	GO:0042981 ~ regulation of apoptosis	6.32E−04
GO:0008104 ~ protein localization	4.68E−10	GO:0055114 ~ oxidation reduction	6.36E−04
GO:0045184 ~ establishment of protein localization	5.39E−10	GO:0042127 ~ regulation of cell proliferation	6.77E−04
GO:0008654 ~ phospholipid biosynthetic process	6.48E−10	GO:0016265 ~ death	6.77E−04
GO:0015031 ~ protein transport	2.46E−09	GO:0043067 ~ regulation of programmed cell death	7.56E−04

Supplementary Table 2. Genes Coexpressed With *SOX15* in a Large Collection of Primary Esophagus, Barrett's Esophagus, and Esophageal Adenocarcinoma Specimens ($r > 0.81$)

Gene	Correlation With *SOX15* Expression
SOX15	1.000
GRHL3	0.981
FAM46B	0.981
ZNF750	0.981
LYPD3	0.981
CAPNS2	0.979
IL20RB	0.978
BNIPL	0.978
ANXA8L2	0.975
GPR87	0.971
TMPRSS11D	0.971
LY6D	0.971
LASS3	0.971
CLCA2	0.971
PKP1	0.971
KRT5	0.971
GSDMC	0.970
TMEM40	0.970
FAM83C	0.970
TP63	0.970
DSC3	0.970
TGM5	0.964
TMPRSS11A	0.964
GJB6	0.964
KLK13	0.964
LYNX1	0.964
SPINK5	0.961
ENDOU	0.961
RNF222	0.961
PRSS27	0.961
KRT78	0.961
CRCT1	0.961
SCEL	0.961
A2ML1	0.961
SLURP1	0.961
c9orf169	0.961
CSTA	0.961
MAL	0.961
KRT6C	0.961
ARHGAP6	0.961
DSG3	0.961
TGM1	0.961
SBSN	0.961
SPRR1B	0.961
CLCA4	0.961
CALML3	0.961
RHCG	0.961
KRT4	0.961
SERPINB13	0.961
GBP6	0.961
NCCRP1	0.961
TMRSS11B	0.961
CNFN	0.961
TGM3	0.961
CRNN	0.961
HSPB8	0.961
SERPINB2	0.961
S100A2	0.961
LGALS7B	0.952
LGALS7	0.952
DUOX1	0.949
DUOXA1	0.949
CSNK1E	0.944
CLIC3	0.942
HOPX	0.940
SERPINB4	0.939
SERPINB3	0.939
ECM1	0.937
Trim29	0.937
SLC39A2	0.937
RAET1G	0.937
AQP3	0.937
TMEM154	0.937
GNA15	0.937
SULT2B1	0.937
ALDH3B2	0.937
EVPL	0.937
GRHL1	0.937
KAZ	0.937
PITX1	0.937
TMEM79	0.937
DENND2C	0.937
VSIG10L	0.937
ZNF185	0.937
PPL	0.937
GJB5	0.937
KRT15	0.937
c10orf99	0.931
EPHX3	0.928
AIF1L	0.928
CRABP2	0.928
PPP1R3C	0.917
ZNF365	0.916
CPA4	0.916
SPINK7	0.916
RNASE7	0.916
LOC643479	0.916

Supplementary Table 2. Continued

Gene	Correlation With *SOX15* Expression
TIAM1	0.912
ARL4D	0.912
LASS4	0.912
IVL	0.912
P2RY1	0.912
DLK2	0.912
ANXA8	0.912
BBOX1	0.911
CYP4F22	0.911
SCNN18	0.911
MUC15	0.911
CWH43	0.911
CALML5	0.909
CST6	0.906
FAM83A	0.904
CDA	0.904
KRT80	0.904
LYPD2	0.901
FGF11	0.899
PPP2R2C	0.899
TLE3	0.881
DOCK9	0.881
PLD2	0.881
PYGL	0.881
BNIP3	0.881
TUBB6	0.881
NDUFA4L2	0.881
BDKRB1	0.881
NDRG4	0.881
CBR3	0.881
SLC22A17	0.881
SRPX2	0.881
FRMD6	0.881
MID2	0.881
EFS	0.881
PARD6G	0.881
c3orf54	0.881
RGMA	0.881
RRAGD	0.881
ANKRD35	0.881
TNFAIP8L3	0.881
ELOVL4	0.881
CRYAB	0.881
GPC1	0.881
ZNF385A	0.881
WDFY2	0.881
NOD2	0.881
PTPN13	0.881
TFAP2C	0.881
CDK5R1	0.881

Supplementary Table 2. Continued

Gene	Correlation With *SOX15* Expression
VSNL1	0.881
MICALL1	0.872
PAK6	0.872
PVRL1	0.872
PVRL4	0.872
ITPKC	0.872
SPTBN2	0.872
SAMD9	0.872
AIM1L	0.872
PLCD1	0.872
NLRX1	0.872
MPZL2	0.872
PRRG4	0.872
PPP1R13L	0.872
URGCP	0.872
XG	0.863
HES2	0.863
c12orf54	0.863
IRX4	0.863
DST	0.863
BNC1	0.863
ARHGEF4	0.858
DMKN	0.855
KLK10	0.855
LTB4R	0.855
CYP2E1	0.853
KCTD1	0.852
ATP13A4	0.849
ATP6V0A4	0.849
RASGRP1	0.849
TRIM6	0.849
SHROOM2	0.849
CLIP4	0.849
KLK12	0.840
S100A8	0.840
S100A9	0.840
KRT6B	0.840
SPRR3	0.840
KRT13	0.840
SPRR1A	0.840
SPRR2A	0.840
NCKAP5	0.833
GPR1	0.833
CDKN2B	0.833
LOC653110	0.833
SFTPD	0.833
FBXO27	0.833
ZNF433	0.824
CA12	0.824
WNT4	0.824

Supplementary Table 2. Continued

Gene	Correlation With *SOX15* Expression
MTSS1	0.824
WDR47	0.824
ELL2	0.824
RORA	0.824
SLC9A9	0.824
SEMA4A	0.824
MAF	0.824
BEX4	0.824
SERPINB8	0.824
ZDHHC21	0.824
ZNF425	0.824
SNX24	0.824
ALDH4A1	0.824
NOTCH2NL	0.824
DUSP22	0.824
MBD2	0.824
C4ORF3	0.824
CNOT1	0.824
CTTNBP2NL	0.824
CUL4B	0.824
ADK	0.824
MOSPD1	0.824
PDZD2	0.824
CAB39P	0.824
ZNF431	0.824
RASAL2	0.824
TMOD3	0.824
ARHGAP10	0.824
DAPP1	0.824
SLC2A6	0.824
ZNF426	0.824
RIT1	0.824
UBE2H	0.824
SPTLC1	0.824
KAT2B	0.824
SECISBP2L	0.824
KIAA1370	0.824
RNF11	0.824
WDR26	0.824
RANBP9	0.824
ABHD5	0.824
YOD1	0.824
SEPT10	0.824
UBE2G1	0.824
SASH1	0.824
GAB1	0.824
PARD3	0.824
RSC1A1	0.824
TPD52L1	0.824
IL34	0.824

Supplementary Table 2. Continued

Gene	Correlation With *SOX15* Expression
HRASLS	0.824
ESPL1	0.824
TRPS1	0.824
KCNG1	0.824
HSPA2	0.824
ABLIM3	0.824
FNDC4	0.824
VAT1	0.824
FLJ11235	0.824
DKK4	0.824
WWTR1	0.824
ATG9B	0.824
PADI3	0.824
SYNPO2L	0.824
TCP11L2	0.824
CCNG2	0.824
GPR110	0.824
SPINK8	0.824
GYS2	0.824
INPP5A	0.824
ILI2A	0.824
UPK1A	0.824
KCNK7	0.824
CYP4B1	0.824
SYNGR1	0.824
OGFRL1	0.824
SLC16A6	0.824
HLF	0.824
DLG2	0.824
CYP11A1	0.824
PTN	0.824
BARX2	0.824
CLDN8	0.824
RHOV	0.824
C1ORF161	0.824
SNX31	0.824
ACPP	0.824
S100A13	0.824
USH1G	0.824
GNG4	0.824
ADAMTSL4	0.824
SLC13A4	0.824
POU3F1	0.824
IL1RN	0.824
IL1F6	0.824
KLK11	0.824
MREG	0.824
ZBED2	0.824
CALB2	0.824
ALOX12	0.824

Supplementary Table 2. Continued

Gene	Correlation With *SOX15* Expression
CRISP3	0.824
EHD3	0.824
ACYP2	0.824
C2ORF54	0.824
KLF8	0.824
KREMN1	0.824
TPRG1	0.824
PAX9	0.824
SUSD4	0.824
DAPL1	0.824
ARSF	0.816
ALOX15B	0.816
C18ORF26	0.816
FMO2	0.816
FAM63A	0.806
SH3GL3	0.806
LY6G6C	0.806
DSG1	0.806
CLDN17	0.806
KPRP	0.806
IGFL1	0.806