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SOX15 governs transcription in human stratified epithelia and a subset of esophageal adenocarcinomas

Rita Sulahian[1,2], **Justina Chen**[1], **Zoltan Arany**[3], **Unmesh Jadhav**[1,2], **Shouyong Peng**[1], **Anil K. Rustgi**[4], **Adam J. Bass**[1,2], **Amitabh Srivastava**[5], **Jason L. Hornick**[5], and **Ramesh A. Shivdasani**[1,2,6]

[1]Department of Medical Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, MA 02215, USA

[2]Department of Medicine, Harvard Medical School, 45 Shattuck Street, Boston, MA 02115, USA

[3]Cardiovascular Institute, Perelman School of Medicine, 3620 Hamilton Walk, University of Pennsylvania, Philadelphia, PA 19104, USA

[4]Division of Gastroenterology, Departments of Medicine and Genetics, Perelman School of Medicine, 3620 Hamilton Walk, University of Pennsylvania, Philadelphia, PA 19104, USA

[5]Departments of Pathology, Brigham & Women's Hospital and Harvard Medical School, 75 Francis Street, Boston, MA 02115, USA

Abstract

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 absence of key squamous-cell factors. As few determinants of stratified epithelial cell-specific gene expression are characterized, it is important to identify the necessary transcription factors.

Methods—We tested regional expression of mRNAs for all putative DNA-binding proteins in the mouse digestive tract and verified esophagus-specific factors in human tissues and cell lines. Integration of diverse data defined a human squamous esophagus-specific transcriptome. We used chromatin immunoprecipitation (ChIP-seq) to locate transcription factor binding sites,

[6]**Corresponding author:** Ramesh A. Shivdasani, MD, PhD, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, MA 02215, Tel: 617-632-5746, Fax: 617-582-7198, ramesh_shivdasani@dfci.harvard.edu.

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Synopsis: 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.

Conflicts of interest: No conflicts of interest exist.

Author contributions: R.S. and R.A.S. conceived and designed the study. R.S. and J.C. performed experiments. Z.A. provided mouse TF libraries and A.K.R. provided cell lines. U.J. helped analyze RNA-seq data. S.P. and A.B. analyzed data from TCGA cancers. A.S. and J.L.H. provided EAC specimens and performed and scored immunohistochemistry. R.A.S. provided overall supervision. R.S. and R.A.S. drafted the manuscript, with contributions from all authors.

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computational approaches to profile transcripts in cancer datasets, and immunohistochemistry to reveal protein expression.

Results—The transcription factor 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 reduced esophageal transcripts significantly and specifically. SOX15 binding is highly enriched near esophagus-expressed genes, indicating direct transcriptional control. *SOX15* and hundreds of genes co-expressed 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.

Keywords

Esophageal gene regulation; Barrett's esophagus; esophageal transcriptome; SOX15 cistrome

Introduction

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 3 to 5 cases per 1,000 person-years (2). Esophageal adenocarcinoma (EAC) arises principally in the setting of BE and the incidence of this cancer in the West increased about 8-fold between 1970 and 2010, with about 18,000 new US 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) 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 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. TP63 regulates differentiation of all stratified epithelia, such as those in the esophagus and skin (10, 11), acting in part through another transcription factor, BNC1 (12). SOX2 controls esophageal differentiation in embryos (13) and growth of adult progenitor cells (14, 15), an activity in which KLF4 and KLF5 also may participate (16). 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.

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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 SOX15 as such a TF and 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, co-expressing representative intestinal and squamous-specific genes within the same tissue. Together, these data identify a novel regulator of stratified epithelial genes and a sub-type of EAC with bi-lineage gene expression.

Results and Discussion

Identification of TFs that are specific to the esophageal epithelium and attenuated in BE

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 (Fig. 1A). Among 1,880 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* (18), underscoring the fidelity of the experimental approach (Fig. 1B). Forty factors and 59 TF genes showed considerably higher expression in esophageal cells, compared to the gastric corpus and the intestine, respectively (Fig. 1A), and 21 TFs were common to the two esophagus-specific groups (Fig. 1B). To exclude variability among mouse strains and to assess specificity relative to non-digestive organs, we measured expression of these 21 mRNAs in 9 diverse tissues from C57BL/6 mice, including the skin. Six TFs gave consistent evidence of high tissue specificity (Fig.1C-D). *Sim2* and *Pax9* showed the greatest specificity, followed by *Sox15* and *Trim29*, which showed some expression in murine skin. Additional data from 65 adult human tissues (19) revealed robust expression of each of these four TF mRNAs in the esophagus, with varying levels in other stratified squamous tissues, such as the tongue, mouth, pharynx, and skin derivatives (Fig. 2A).

To determine if 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 non-dysplastic BE; and CP-B and CP-C, which represent BE with high-grade dysplasia. This cell line series replicates disease progression (21), with reduced levels of multiple keratin mRNAs (Fig. 2B). We observed a concomitant decline in *SOX15* and *TRIM29* levels, matching or exceeding that of *TP63* mRNA, with little variance in the other factors (Fig. 2C). 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 less *SOX15* and *TRIM29* mRNAs in primary BE, compared to adjacent normal esophageal mucosa (Fig. 2D). Finally, we used Oncomine tools (22) to analyze mRNA data from an independent series of 28 frozen human normal esophagus and 15 frozen BE biopsy specimens (23). Levels of *PAX9*,

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S*OX15* and *TRIM29* were uniformly high in normal esophagus and attenuated in BE specimens (Fig. 2E).

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 (Fig. 2C), and although TRIM29 has a putative DNA-binding domain, its role in transcriptional regulation is poorly defined and uncertain (24). By contrast, SOX proteins control differentiation of diverse tissues, often in conjunction with other family members (25), 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 (26-28) and we proceeded to investigate its functions.

SOX15 depletion affects genes specific to stratified epithelium

To test if 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 (19) (see Fig. 2A) and identified 362 genes that express at >3-fold higher levels ($p<0.05$) in the esophagus than in any of 7 diverse tissues, including glandular stomach, from the same collection of postmortem samples (Fig. 3A). Second, we identified 300 genes with >3-fold higher mRNA levels ($p<0.05$) 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 (Fig. 3A). 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 if 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 (Fig. 2B-C) and they tolerate lentiviral infection and drug selection. Because *SOX15* depletion retarded CP-A cell growth and survival, we harvested cells 72 h post-infection, when they appeared healthy but *SOX15* mRNA levels were appreciably reduced (Fig. 3B). RNA-seq analysis showed reduced and increased levels of 2,950 and 717 transcripts, respectively, compared to cells treated with a non-specific shRNA (Fig. 3C). In agreement with the deficit in cell growth, these genes were enriched for Gene Ontology terms related to the cell cycle (Suppl. Table 1). More importantly, genes reduced in *SOX15*-depleted cells included 26.4% of the human esophagus-specific “union” transcriptome, compared to 15.34% overlap with multiple sets of 2,950 random genes expressed in CP-A cells (Fig. 3D, $P<0.0001$). Correspondence was even higher for the esophagus-specific “intersection” transcriptome, where 31.5% of genes were reduced in *SOX15*-depleted cells, compared to 14.68% of random genes ($P<0.0001$). SOX15-dependent genes were highly enriched for functions related to stratified epithelia (Fig. 3D).

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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 (Suppl. 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 if SOX15 might regulate dependent genes directly, we used chromatin immunoprecipitation with high-throughput sequencing (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 5,000 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 (Fig. 4A). 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 (Fig. 4A). GREAT analysis (29) 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 (Fig. 4B). 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 to about 5% of comparable numbers of random genes ($P<0.0001$, Fig. 4C). Gene Ontology terms related to stratified epithelia were further enriched among SOX15-bound genes (Fig. 4C). Most importantly, genes affected by SOX15 depletion in CP-A cells were highly enriched for nearby SOX15 binding, compared to random gene sets of equal size ($P<0.0001$) and SOX15-bound genes reduced in SOX15-depleted cells far outnumbered genes that were increased (Fig. 4D-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* illustrate SOX15 occupancy at putative *cis*-regulatory sites and reduced expression in SOX15-depleted cells (Fig. 4F).

SOX15 in human EAC

SOX15 is expressed highly in normal human esophagus, but not in the BE cell lines CP-B and CP-C (Fig. 2C) or in areas of intestinal metaplasia *in vivo* (Fig. 2D-E). To our surprise, RNA expression data from a large collection of frozen primary esophagus, BE and EAC biopsy specimens (23) revealed high *SOX15* mRNA levels in up to 1/3 of EACs (Fig. 5A, left; note that all samples in this study, including EAC, were frozen biopsy specimens). Moreover, at least 317 transcripts that are strongly co-expressed with *SOX15* in the normal esophagus ($r>0.81$, Suppl. Table 2) were also present in the same EAC specimens (Fig. 5A 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 co-expressed with *SOX15*, (Fig. 5B). The canonical intestinal marker *CDX2* and its co-expressed genes ($r>0.81$) were expressed in many

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SOX15^{+} and also in *SOX15*$^{-}$ specimens (Fig. 5A, right), revealing co-expression of esophageal and intestinal genes in some cases. Moreover, 21.6% of genes co-expressed with *SOX15* in this analysis showed SOX15 binding within 50 kb in CP-A cells, compared to ~6% of random genes (P<0.0001, Fig. 5C top), which implies that many of these genes are direct transcriptional targets. Indeed, the effects of SOX15 depletion were significantly greater on these genes than on random sets of genes expressed in CP-A cells (P<0.017, Fig. 5C bottom). These features collectively suggest direct SOX15 regulation of many esophagus-restricted genes that are silent in BE and reactivated in up to 1/3 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 non-malignant cells were meticulously minimized (30). Cancers of the gastro-esophageal (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 co-expressed with *SOX15* in normal esophageal epithelium (Suppl. Fig. S1A-B). Transcripts specific to the squamous esophageal epithelium were thus again evident in a fraction of EACs. To determine if 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 (Fig. 5D).

Finally, we examined 99 separate EACs by immunohistochemistry (IHC) on resection specimens. Because several antibodies failed to detect SOX15, we used KRT5 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 (23) (Fig. 6A, 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 co-expressed nuclear CDX2 (Fig. 6B). Levels of KRT5 were variable (Fig. 6B) but did not correlate with tumor grade or other pathological 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 (Figs. 6C-D show examples from 2 different cases). Co-expression 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 (Figs. 5A and 6A), we used IHC 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 (Fig. 6E). These findings extend previous reports of absent expression of stratified epithelium-specific keratins in BE (31-33) and low-level expression of squamous cell products in EACs (34). Our delineation of a squamous cell- restricted transcriptome (Fig. 3A), coupled with re-analysis of published RNA expression data (Fig. 5A-B) and investigation of additional cases by IHC (Fig. 6B-D),

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reveals for the first time the extent and breadth of an aberrant stratified-cell program in EACs.

Implications for esophageal squamous differentiation and EAC

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 understanding of esophageal differentiation and pathology. The lack of overt esophageal defects in *Sox15* mutant mice (35, 36) is compatible with the considerable known redundancies among SOX-family TFs (25).

There is much debate whether BE, and particularly EAC, originates 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, 37). 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 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* are highly expressed in these cells (Fig. 2C) and in BE biopsy specimens (Fig. 2D-E). Moreover, >300 esophageal genes, including *SOX15*, are active in up to 30% of human EACs (Fig. 5A), with intestinal genes such as *CDX2* often co-expressed in the same glands as esophageal genes (Fig. 6C-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 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 (38, 39), 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 clinical and genetic correlates of these EACs showing bi-lineage gene expression and to determine if they reflect a distinctive pathophysiology or harbor unique therapeutic vulnerabilities.

Materials and Methods

All authors had access to the study and reviewed and approved the final manuscript.

Tissue preparation and TF 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

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esophagus was treated with 0.1% Collagenase-Dispase (Roche, catalog no. 11097113001) in phosphate-buffered saline (PBS) for 15 min at 37°C, whereas stomach and duodenum were incubated in 1 mM EDTA in PBS at 37°C. To determine relative transcript levels (Fig. 1A-C), we used qRT-PCR and a library containing oligonucleotide primers specific to 1,880 known and putative TFs (40). Tissue-specific TFs were identified using the comparative C_T method (41). To further determine tissue specificity (Fig. 1D), other whole organs were harvested from adult C57BL/6 mice.

Cell lines

CP-A (KR-42421), CP-B (CP-52731) and CP-C (CP-94251) cells (American Type Culture Collection) were cultured in MCDB-153 medium (Sigma, catalog no. M7403) supplemented with 0.4 μg/ml hydrocortisone, 20 ng/ml recombinant human Epidermal Growth Factor (Sigma, catalog no. E9644), 8.4 μg/L cholera toxin (Sigma, catalog no. H0135), 20 mg/L adenine (Sigma, catalog no. A2786), 140 μg/ml bovine pituitary extract (Sigma, catalog no. P1476), ITS Supplement (Sigma, catalog no. I1884, 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) supplemented with bovine pituitary extract and recombinant human epidermal growth factor (Gibco). Soybean trypsin inhibitor (Sigma) was used to quench trypsin activity during cell passage.

Gene analyses

Figs. 2A, 2D-E, 5A and 6A show analyses of relative mRNA expression levels from published studies of 65 adult human tissues (19), of human esophageal biopsy specimens (8), and of human normal esophagus, BE and EAC samples (23). The data were re-analyzed with respect to *SOX15* using Oncomine tools (Compendia Bioscience, https://www.oncomine.com), considering all samples in each dataset. Genes significantly associated with *SOX15* (Fig. 5A) were ranked on the basis of correlation values. Enriched Gene Ontology terms (Figs. 3A, 3D, 4B, 4C, 5B) were determined using DAVID tools (http://david.abcc.ncifcrf.gov/). We examined processed RNA-seq data from a TCGA study on stomach cancer (30), first isolating 30 CIN^+ tumors arising at the G-E junction or gastric cardia for unsupervised clustering (Suppl. Fig. 1). To this group we applied hierarchical clustering (using hclust from the R package; http://cran.r-project.org) on the 1,000 most variable transcripts normalized according to expression z-scores, followed by a second hierarchical clustering on the set of 317 genes co-expressed with *SOX15*. To assess specificity of *SOX15* overexpression in tumors of the gastric cardia (Fig. 5D), we compared with RNA-seq data from TCGA studies on colon (42) and distal gastric adenocarcinomas (30).

Experimental RNA analyses

Total RNA was isolated using Trizol (Invitrogen), treated with the RNeasy Mini Kit (Qiagen), and DNA was digested using Turbo DNA-Free (Ambion). For qRT-PCR analysis (Figs. 1A-C, 2B-C, 3B), 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). RNA-seq libraries (Fig. 3C, full dataset is deposited in the Gene Expression Omnibus with accession number GSE62909) were prepared from 300

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ng of total RNA using TruSeq RNA Sample Preparation kits (Illumina) and 75-bp single-end sequences were obtained on a NextSeq 500 instrument (Illumina). Reads were aligned to human genome build Hg19 using TopHat v2.0.6. Expression levels of transcripts in duplicate samples were calculated as fragments per kb per 10^6 mapped reads (FPKM) using Cufflinks v2.0.2 and differential expression was determined using CuffDiff (43). 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

Cells were infected with lentiviruses generated from the pLKO.1 vector (Open Biosystems) carrying either a *SOX15*-targeting shRNA (TGCCTGGCAGCTATGGCTCTT) or a control, non-specific 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 (Addgene, catalog no. 24129) together with cassettes for the T2A sequence, biotin, and BirA-V5 (gift of Ben Ebert, Brigham & Women's Hospital, Boston, MA).

Chromatin immunoprecipitation (ChIP) and ChIP-seq

Cells were cross-linked with 2 mM disuccinimidyl glutarate (DSG, Pierce, catalog no. 20593) in PBS for 45 min, followed by 10 min with 1% formaldehyde (Pierce) in PBS, at room temperature. ChIP and ChIP-seq were performed as described previously (44), using 30 μL slurry of streptavidin-conjugated magnetic beads (Invitrogen, catalog no. 65601). 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 (IGV) (45). Functions of genes within 50 kb of SOX15 occupancy were determined using GREAT (29). ChIP-seq data are deposited in the GEO database with accession number GSE62909.

Immunohistochemistry

4-μm-thick tissue paraffin sections were baked overnight at 37°C, deparaffinized in xylenes, rehydrated, and peroxidase activity was blocked with 1.5% H_2O_2 in methanol for 10 min. Slides were treated with 0.01 M citrate buffer, pH 6.0 in a pressure cooker at 120°C for 30 min 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 min, followed by Dako Envision+ Mouse (Dako K4007) secondary Ab for 30 min, and developed with 3,3'-diaminobenzidine (Dako). Sections were then incubated with mouse KRT5 Ab (clone XM26, Leica NCL-L-CK, 1:500) for 40 min, followed by PowerVision AP mouse (Leica, catalog no. PV6110) secondary Ab for 30 min, 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 min, and developed with 3,3'-diaminobenzidine (Dako).

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Supplementary Material

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Acknowledgments

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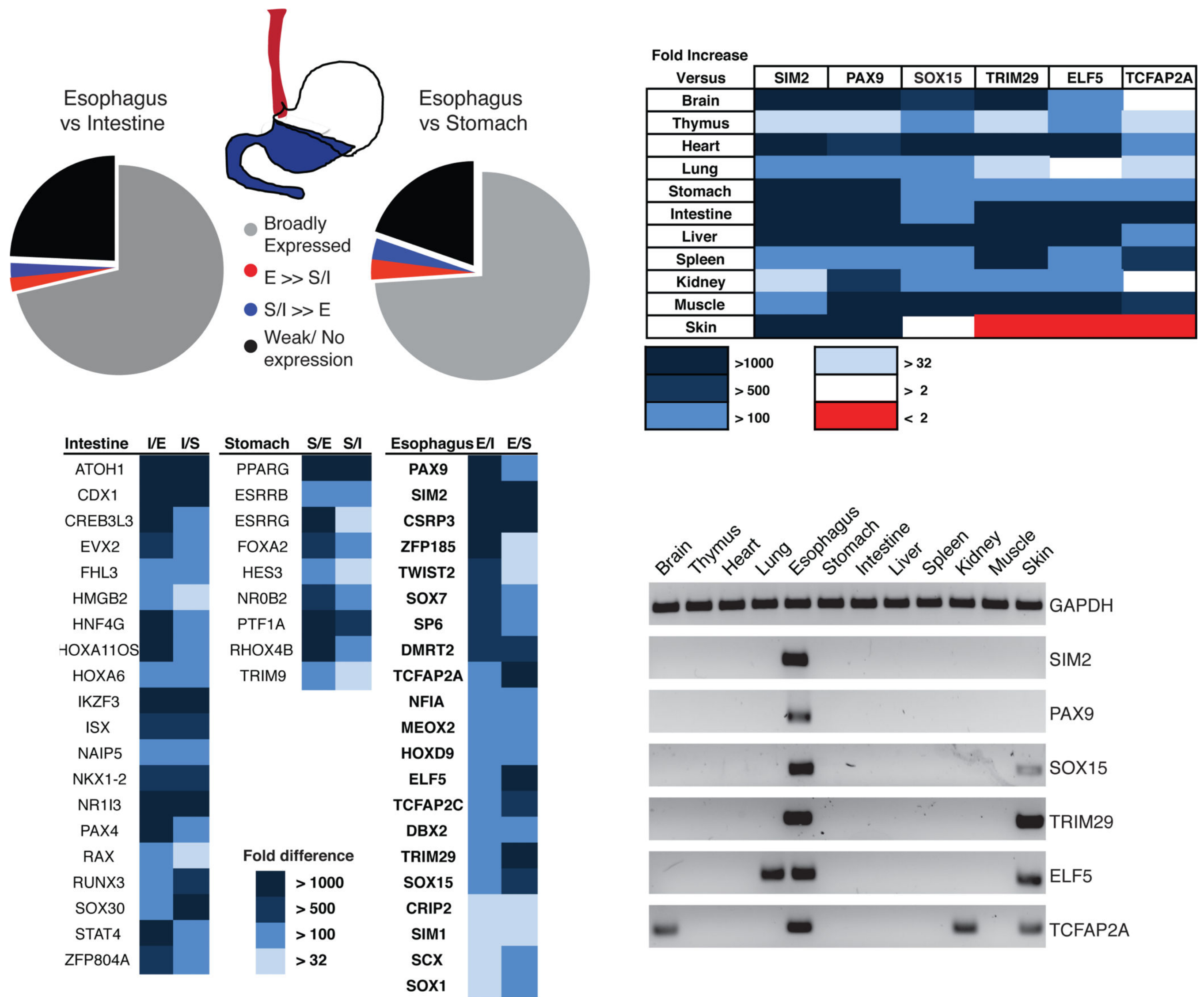

Figure 1. Differential TF expression in the normal mouse gut and other tissues

(A) Distribution of all TFs in wild-type mouse digestive epithelia, as revealed in a qRT-PCR screen. Expression of 1,880 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 6 most highly esophagus-specific TF mRNAs in 12 adult mouse organs, showing selective expression in the esophagus and of some factors in the skin.

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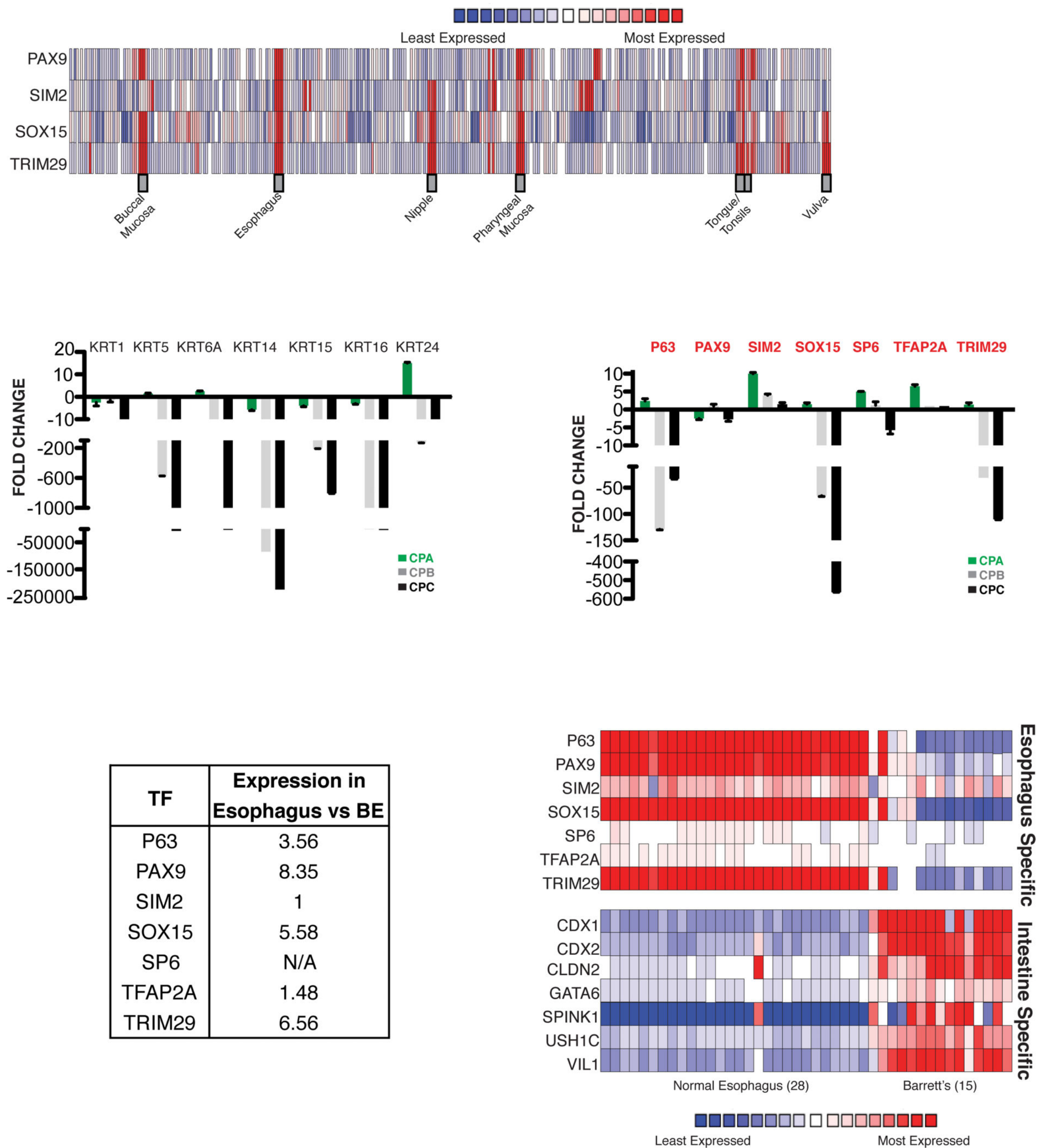

TF	Expression in Esophagus vs BE
P63	3.56
PAX9	8.35
SIM2	1
SOX15	5.58
SP6	N/A
TFAP2A	1.48
TRIM29	6.56

Figure 2. Differential TF expression in normal and metaplastic human esophagus

(A) Expression profiles of *PAX9, SIM2, SOX15* and *TRIM29* in 65 human organs. Data from necropsies (19), analyzed using Oncomine tools (22), 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 BE cell line series (CPA, CPB and CPC) with increasing dysplasia. Results of qRT-PCR 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 from Ref. (23) were analyzed using Oncomine tools.

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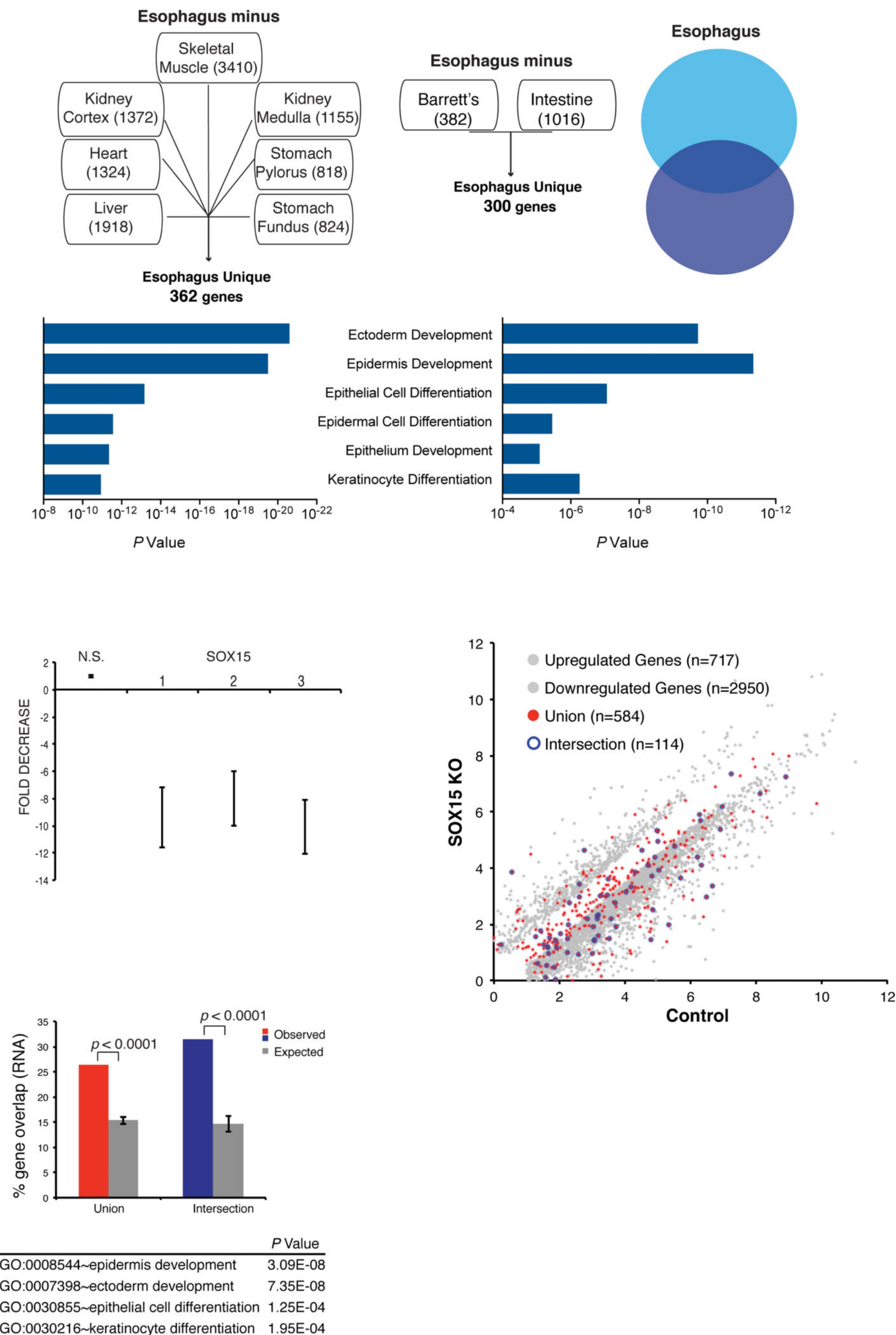

	P Value
GO:0008544~epidermis development	3.09E-08
GO:0007398~ectoderm development	7.35E-08
GO:0030855~epithelial cell differentiation	1.25E-04
GO:0030216~keratinocyte differentiation	1.95E-04

Figure 3. Impact of SOX15 depletion on esophageal gene expression

(A) Delineation of the human esophageal transcriptome. mRNAs expressed in human esophageal necropsy specimens (left) were compared against transcripts from 7 other postmortem organs (19) and mRNAs present in fresh esophageal biopsy specimens (right) were compared against transcripts from fresh 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* <0.0001, χ^2 test). The top Gene Ontology (GO) terms in each case are highly related to

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stratified epithelia. **(B)** *SOX15* mRNA depletion in 3 representative experiments in which CPA cells were infected with lentiviruses carrying *SOX15*-specific or a non-specific (NS) 21-bp shRNAs. Knockdown efficiency, assessed by qRT-PCR 72 h 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, non-specific (x-axis) shRNAs. Grey dots mark differential expression ($\log_2$ >1.5-fold, q <0.05) and 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 to 5 random sets of equal numbers of genes expressed in CP-A cells (grey bars). The table lists GO terms enriched among SOX15-dependent genes.

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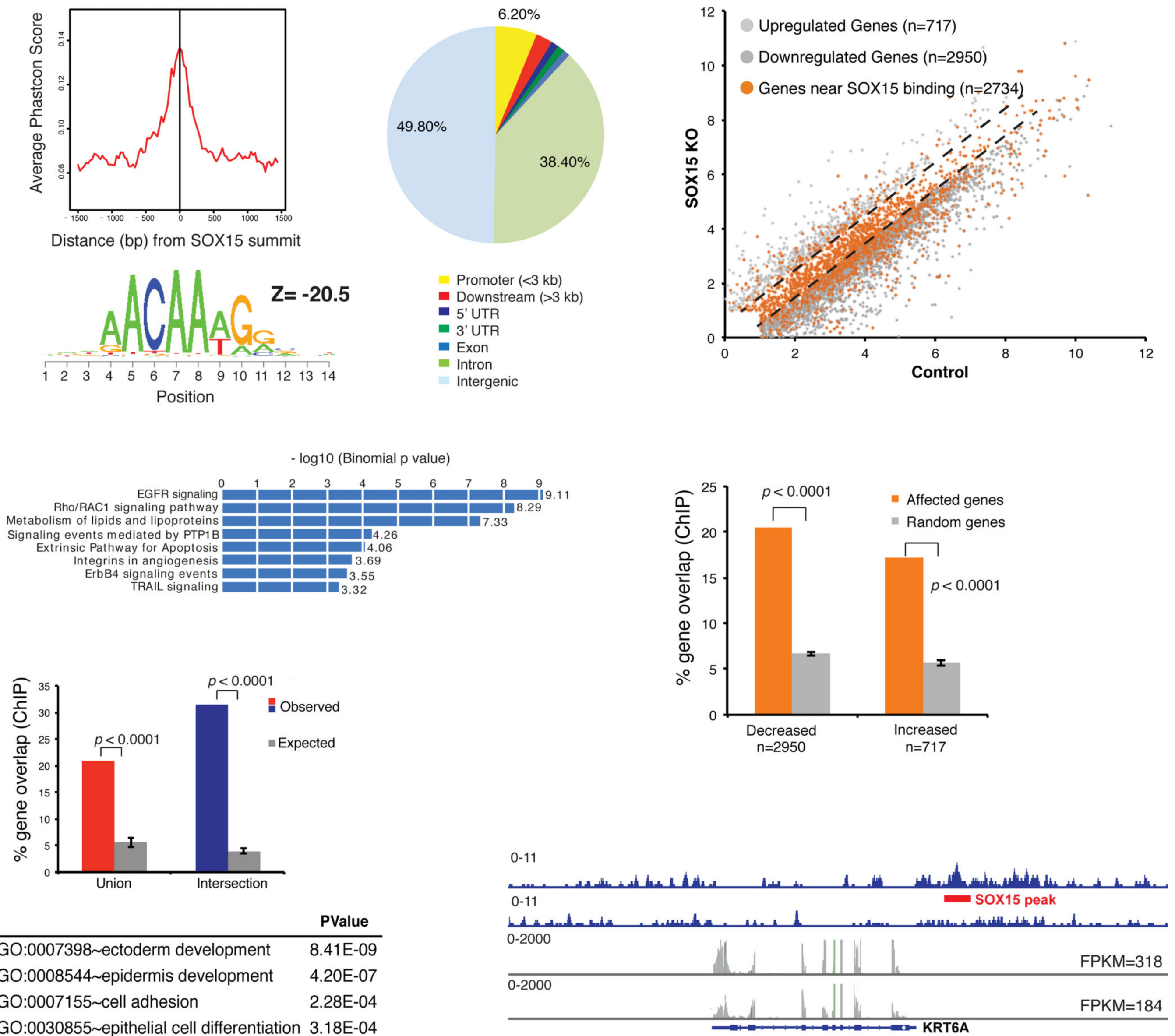

	PValue
GO:0007398~ectoderm development	8.41E-09
GO:0008544~epidermis development	4.20E-07
GO:0007155~cell adhesion	2.28E-04
GO:0030855~epithelial cell differentiation	3.18E-04

Figure 4. Genome-wide SOX15 occupancy and gene dependence in human esophageal cells

(A) Summary of 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 4,864 identified binding sites. SOX15 mainly binds DNA far from promoters. **(B)** GO terms enriched among the 2 nearest genes within 50 kb of SOX15 binding sites, determined using GREAT (29). **(C)** Percentages of esophagus-specific genes (as determined in Fig. 2A, red: union, blue: intersection set) that bind SOX15 within 50 kb of the transcription start site (TSS), compared to 5 random gene sets of equivalent size (grey bars, $P<0.0001$). 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, $q<0.05$, as in Fig. 2B). Dashed lines demarcate genes unaffected by SOX15 loss. **(E)** Genes reduced or increased in SOX15-depleted cells are significantly enriched for nearby SOX15

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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.

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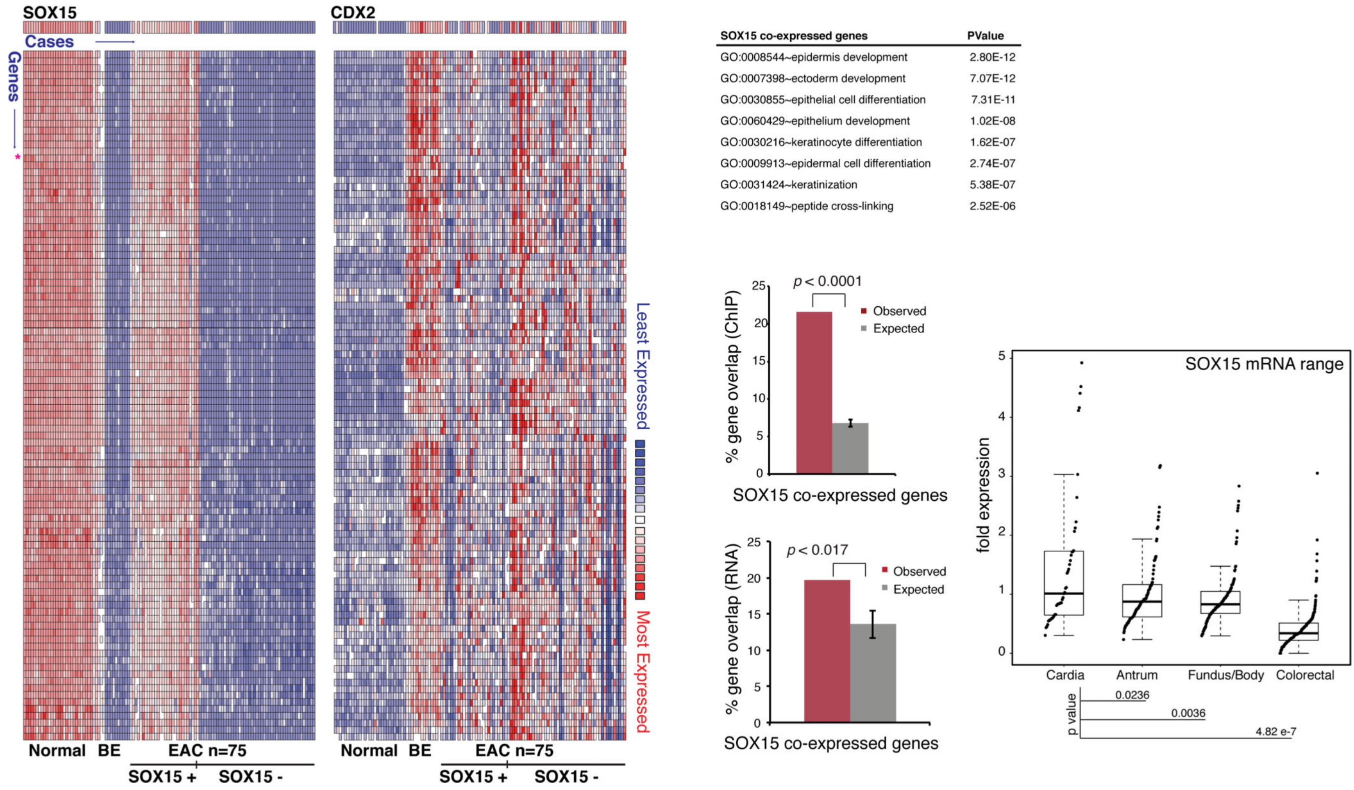

Figure 5. SOX15 expression in esophageal adenocarcinomas

(A) Gene co-expression profiles for *SOX15* (left) and *CDX2* (right) in a large collection of normal, BE and EAC epithelium (23). 317 transcripts correlated strongly (r >0.81) with *SOX15* mRNA levels in normal esophagus and in approximately 1/3 of 75 EACs in this series. The 100 most highly correlated genes are shown. **(B)** GO term enrichment among these 317 *SOX15*-coexpressed genes. **(C)** Top: Fraction of *SOX15* co-expressed genes showing SOX15 occupancy (Observed) within 50 kb, compared to the fraction expected for appropriate random gene sets of equal size. Bottom: Fraction of *SOX15* co-expressed genes affected by SOX15 depletion (Observed), compared to the fraction expected among random gene sets of equal size. **(D)** Ranges of *SOX15* mRNA expression extracted from RNA-seq data on TCGA collections of cancers of the gastric cardia, fundus/body and antrum (30), or colon and rectum (42). Significance of differences was determined by the t-test.

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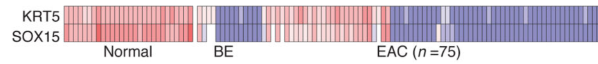

Total EAC Cases	KRT5 positive %	CDX2 positive %	Both expressed %
99	19	83	18

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	+

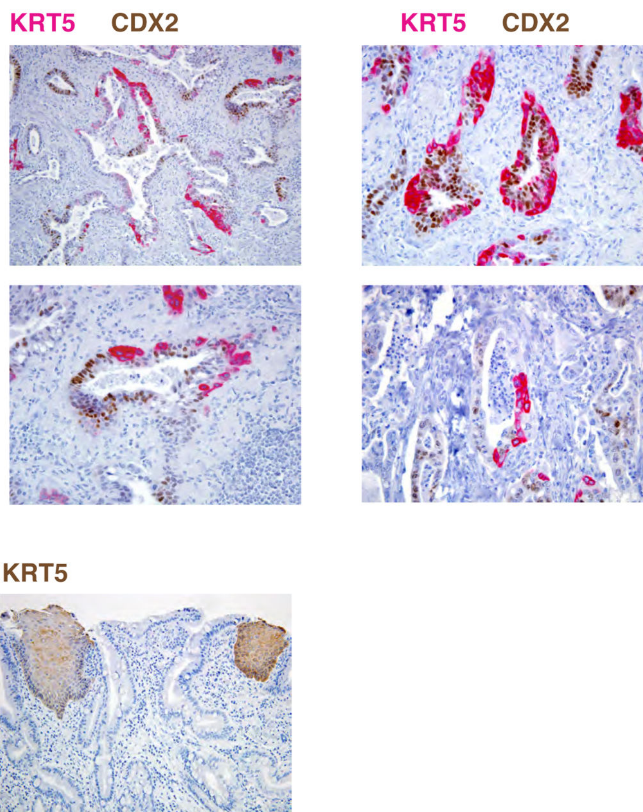

Figure 6. Bi-lineage gene expression in a subset of EACs

(A) High correlation (*r*=0.97) of *SOX15* and *KRT5* mRNAs in normal esophagus, BE, and EAC, validating *KRT5* as a proxy for *SOX15* and other stratified epithelium-specific genes. **(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, 200X; Bottom, 400X.

(E) Absence of KRT5 immunostaining in areas of BE. Adjoining areas of normal stratified epithelium provide a positive control and contrast.

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