Barrett’s esophagus in gastrointestinal reflux patients constitutes a columnar epithelium with distal characteristics, prone to progress to esophageal adenocarcinoma. HOX genes are known mediators of position-dependent morphology. Here we show HOX collinearity in the adult gut while Barrett’s esophagus shows high HOXA13 expression in stem cells and their progeny. HOXA13 overexpression appears sufficient to explain both the phenotype (through downregulation of the epidermal differentiation complex) and the oncogenic potential of Barrett’s esophagus. Intriguingly, employing a mouse model that contains a reporter coupled to the HOXA13 promoter we identify single HOXA13-positive cells distally from the physiological esophagus, which is mirrored in human physiology, but increased in Barrett’s esophagus. Additionally, we observe that HOXA13 expression confers a competitive advantage to cells. We thus propose that Barrett’s esophagus and associated esophageal adenocarcinoma is the consequence of expansion of this gastro-esophageal HOXA13-expressing compartment following epithelial injury.
Barrett’s esophagus (BE) and gastric intestinal metaplasia (IM) are important risk factors for adenocarcinoma of the esophagus and stomach. In the esophagus, the chronic inflammation associated with gastroesophageal reflux disease (GERD) is believed to lead to Barrett’s esophagus (BE), a crypt-structured columnar epithelium with distal gastrointestinal (GI)-tract characteristics, located just above the gastro-esophageal junction (GEJ). BE is a precursor lesion for esophageal adenocarcinoma comparatively to other sites of the ileum and the proximal esophagus represent relatively high-risk regions for carcinoma (EAC), a disease which has shown a strong increase in incidence in the past decades. Analogously, *H. pylori*-infection can degenerate into atrophic gastritis and gastric IM, which in turn can progress into gastric cancer, the third leading cause of cancer-related death. Similarly, while absolute risk is low, heterotopic tissues in Meckel’s diverticula and gastric inlet patches of the proximal esophagus represent relatively high-risk regions for adenocarcinoma comparatively to other sites of the ileum and proximal esophagus, respectively. Therefore, a deeper understanding of the biology of BE and gastric IM is necessary for designing rational avenues for the prevention and treatment of GI cancers.

BE is characterized by the presence of cells with a caudal intestinal phenotype at a rostral location. Therefore, dysregulation of positional specification is likely involved in the etiology of BE. Regulation of rostral-caudal patterning of specialized tissue in embryology and adulthood is to a large extent dependent on the concerted action of two evolutionary highly conserved gene systems, the Caudal-related Homeobox (CDX) transcription factor gene family and the genes of the Homeobox (HOX) cluster. A substantial research effort has been invested in investigating the role of CDX genes in positional misspecification in BE. However, these efforts have not yielded convincing evidence that these genes are the principal mediators of the distal phenotype in this disease. Intriguingly, however, a microarray-based gene expression study of BE suggested potential misregulation of the HOX gene family in BE. HOX genes are linked to morphological transformations and neoplasia. Four clusters of HOX genes, HOXA to HOXD, have been defined. The 3′ to 5′ sequence of HOX gene paralogues corresponds to the sequence in which they act along the rostrocaudal axis. This property is termed collinearity and links clustering to function. Previously, a HOX expression gradient was found along the murine embryonic gut. Ectopic HOX expression in mice can alter intestinal differentiation. A HOX gradient along the adult human gut has also been reported, but that study involved pooling full thickness gut specimens, limiting data interpretation. Nevertheless, we feel that there is sufficient evidence to prompt exploring the function of HOX gene expression with respect to positional identity in physiology and pathology of the GI-tract in general and in BE in particular.

Here, we show that single cells of the upper GI tract express the distal gene HOXA13, that their number is upregulated in BE and that HOXA13 conveys phenotypic metaplasia and increases proliferation.

Of all HOXA paralogues, expression of HOXA13 was highest and restricted to the colon (Fig. 1a). HOXA13 expression is regulated by LncRNA HOTTIP, which is located 5′ to HOXA13. Accordingly, HOTTIP and HOXA13 share a similar expression pattern (Supplementary Fig. 1a). For HOXD, all paralogue genes have increased expression in the distal colon, while HOXC expression is mainly localized in the proximal and ileal regions. Thus, HOX gene expression is linked to positional identity for the BE/HOX paralogues.

Subsequently, we addressed the question as to whether GI HOX coding is already present at the GI stem cell stage, or is established only upon the formation of differentiated derivatives. For this, we used publicly available data published by Wang et al. which contains the mRNA expression of human stem cells isolated from the GI-tract and either cultured as stem cells or differentiated in an air-liquid interface (ALI). Analysis of this data shows that HOX gene expression patterns in stem cell and ALI cultures are similar. HOXA and B cluster genes have a significantly higher expression in the large intestine as compared to the small intestine, in particular 5′ HOXA genes including HOXA13 (Fig. 1d for HOX genes, for clusters HOXB, C, and D see Supplementary Fig. 3). No clear regulation of the HOXC or D clusters is seen in this dataset, with exception of an upregulation of HOXC10 in the large intestine. Hence, HOX coding is an inherent feature of the location-specific stem cell and is maintained in its derivatives.

**HOXA13 in BE, GI heterotopias and GI cancers.** As positional phenotype is linked to HOX status in physiology, we subsequently characterized HOX mRNA expression in several metaplastic tissues known to assume the morphological phenotype of other intestinal locations, as well as their sequelae. BE shows upregulation of HOXA10, 11, and 13, and HOXB 6, 7, 9, and 13 mRNA by qPCR when compared to the normal squamous esophagus (Fig. 1b and Supplementary Fig. 4), which closely resembles colonic HOXA and B expression patterns. High 5′ HOXA gene expression is also present in columnar-lined esophagus without goblet cells (CLE; a BE-related condition), esophageal adenocarcinoma (EAC), and IM of the stomach (Fig. 1b, c). In accordance with a regulatory role for HOTTIP on HOXA13 expression, we find that HOTTIP is also overexpressed in BE, and correlates with HOXA13 expression patterns (Supplementary Fig. 5a–c). HOTAIR, a LncRNA located in the HOXC cluster and associated with chromatin reprogramming in cancer progression is upregulated as well (Supplementary Fig. 5d–f). We concluded that BE, EAC and various metaplasias with caudal histomorphological characteristics have HOXA and HOXB expression patterns typical of the caudal GI-tract, with upregulation of HOXA13 expression being the prominent feature. Heterotopias, namely the gastric inlet patch in the proximal esophagus and heterotopia of the Meckel’s diverticulum, are tissues which have a physiological appearance, but are normally found in a different location. Both these heterotopias are characterized by abundant HOXA13 mRNA expression (Fig. 1c), although intriguingly the direction of epithelial metaplasia for Meckel’s diverticulum is of an anterior rather than posterior phenotype, indicating an exception from the pattern in case of Meckel’s diverticulum. One of the existing hypotheses on the cell of origin of BE states that BE may arise from cells with progenitor properties that are able to give rise to a variety of cell types. To investigate whether aberrant HOX gene expression in BE is established at the level of the epithelium-specific stem cell, we interrogated the publically available data of Yamamoto
Fig. 1 HOXA cluster gene expression shows collinearity along the adult gastrointestinal tract but is deregulated in Barrett’s esophagus (BE), various metaplasias and esophageal adenocarcinoma (EAC). **a** HOXA cluster genes are collinearly expressed along the gastro-intestinal (GI)-tract of adult humans (n = 3). Numbers represent mRNA fold changes relative to the esophagus and thus can be compared within each HOXA paralogue member but not between them. **b** HOXA cluster gene expression in the squamous esophagus of BE patients (n = 13), columnar lined esophagus (CLE) (n = 14), BE (n = 13), EAC (n = 12), and gastric intestinal metaplasia (IM) (n = 12) is characterized by an upregulation of 5′ HOXA genes. Numbers represent mRNA fold changes relative to the esophagus of healthy individuals. **c** HOXA13 expression quantified by qPCR in BE, CLE, IM of the stomach, and heterotopias along the GI tract with their corresponding physiological epithelia. Squamous epithelium (SQ) Barrett’s and BE are derived from the same person (n = 13). Gastric inlet patch (GIP; n = 5); healthy control (HC) squamous esophagus (n = 12; CLE (n = 14); BE (n = 13); EAC (n = 12); stomach (n = 14); gastric IM (n = 12); ileum (n = 6); Meckel’s diverticulum (MD) with gastric heterotopia (n = 14), and colon (n = 9). Median ± IQR, *p < 0.05; **p < 0.01; ***p < 0.001. For esophagus, Kruskal–Wallis test with Dunn’s multiple comparisons test (SQ healthy vs. GIP, p = 0.015; SQ healthy vs. CLE, p < 0.0001; SQ healthy vs. BE, p < 0.0001; SQ healthy vs. EAC, p = 0.0009). For stomach and ileum, Mann–Whitney test (two-tailed), p < 0.0001. **d** HOXA cluster genes, in particular 5′ HOXA genes including HOXA13, have a higher expression in the large intestine (n = 3 in technical duplicate) compared to the small intestine (n = 3 in technical duplicate), in both stem cells (left panel) and differentiated cells (right panel). Normalization was performed by setting mRNA expression to 1 for the small intestine. *p < 0.05; **p < 0.01; ***p < 0.001. This figure includes no estimate of variance as the empirical Bayes-moderated two-sided t-statistic was used which does not generate a standard error. **e** 5′ HOXA cluster gene expression in BE is higher compared to the squamous esophagus in stem cell and air-liquid interface (ALI) differentiated cultures. n = 12 (BE) versus n = 2 (squamous esophagus) in technical duplicates are depicted for stem cell cultures and n = 1 each for ALI differentiated samples in technical duplicates. Normalization was performed by setting mRNA expression to squamous esophagus. *p < 0.05; **p < 0.01; ***p < 0.001. This figure includes no estimate of variance as the empirical Bayes-moderated two-sided t-statistic was used which does not generate a standard error. **f** Deregulation of HOXA13 expression in gastrointestinal tract pathology as evaluated with RNA in situ hybridization in clinical samples. HOXA13 is upregulated in IM and heterotopia and downregulated in pyloric and Paneth cell metaplasia in the colon. One sample of each tissue type was analyzed. **g** Downregulation of HOXA13 expression (corrected for peptidylprolyl isomerase B - PPIB expression) relative to adjacent non-metaplastic tissue, was observed for Paneth cell metaplasia (n = 5; FC 0.59; p = 0.0003) and pyloric metaplasia (n = 5; FC 0.22; p = 0.0001) (lower panels). Unpaired t-test (two-tailed). Representative images of hematoxylin and eosin (HE) staining, HOXA13 RNA-scope, and PPIB reference gene RNA-scope of Paneth cell metaplasia (from the colon) present in two glands to the bottom right (upper panels) and pyloric metaplasia (from the colon) in the top left two glands (middle panels) are shown.
et al.\textsuperscript{15,18}. HOX gene expression patterns in squamous esophageal and BE stem cells as well as their respective ALI-differentiated derivatives were retrieved. HOX gene expression in stem cell cultures from these locations is similar to their ALI-differentiated counterparts (Fig. 1e, Supplementary Fig. 3). In BE stem cells, an upregulation of $5^{th}$ HOXA genes (Fig. 1e) as well as HOXB6, 7, 13, and HOXC10 is seen (Supplementary Fig. 3), reaching levels similar to those observed in the colon. Thus, alternative HOX coding associated with BE is established at the epithelium-specific stem cell level and is maintained in derivatives of the stem cells involved.

According to the collinearity theory, a paralogue group 13 member is more likely to confer the distal characteristics seen in BE as compared to more anterior paralogue group members\textsuperscript{18}. Of the paralogue group 13 members, HOXA13 and HOXB13 are overexpressed in BE, with HOXA13 showing much higher expression compared to HOXB13 in BE, EAC, and IM of the stomach (Supplementary Fig. 4). Therefore, while HOX genes such as HOXA11, B6, B9, and B13 are also potentially interesting candidates, what we chose to focus on the HOXA13 gene for further in-depth analysis of different metaplastic tissues. As immunohistochemistry for HOXA13 was unsuccessful, (two anti-HOXA13 antibodies were tested, but lacked specificity) we resorted to in situ hybridization (ISH) for HOXA13 to further confirm the observed atypical expression of this gene in different tissues (examples shown in Fig. 1f). Metaplasia is found throughout the GI-tract. While BE and IM acquire a more distal phenotype, distally located colonic pyloric and Paneth cell metaplasia, related to inflammatory bowel disease, acquire a more rostral phenotype\textsuperscript{20}. Accordingly, downregulation of HOXA13 expression (corrected for PPIB expression as a reference gene) relative to adjacent non-metaplastic tissue, was seen for these tissues (Fig. 1g, again supporting a role for HOXA13 in positional identity).

**Binary regulation of HOXA13 expression.** To study in more detail which of the cells in the healthy GI-tract express Hoxa13, we employed a murine model in which the endogenous mouse Hoxa13 promoter drives the expression of a Hoxa13-GFP fusion protein. Within the epithelial compartment, the proximal expression border is located at the transition from the distal to the proximal colon as can be seen from fluorescent images and images of anti-GFP IHC staining (Fig. 2a, b and Supplementary Fig. 6a–d for bigger overview images). This proximal expression border seems to be crypt-clonal, with some crypts expressing Hoxa13 and others not (see arrows in Fig. 2b and close-up in Fig. 2c). Functional consequences of this clonality are unknown and, while beyond the scope of the present manuscript, present an interesting biological question. The distal Hoxa13-GFP signal for PPIB expression as a reference gene) relative to adjacent non-metaplastic tissue, was seen for these tissues (Fig. 1g, again supporting a role for HOXA13 in positional identity).

**Individual Hoxa13/HOXA13-positive cells in the upper GI tract.** No significant expression of HOXA13 mRNA was seen in the squamous esophagus of BE patients by qPCR (Fig. 1c, e), suggesting that GERD does not provoke HOXA13 expression per se. Indeed, when two primary immortalized squamous esophageal cell lines (EPC2-hTERT and HET-1A) were exposed to either bile or acid, only minor effects on HOXA13 expression were observed (two to fourfold from a low baseline expression; Fig. 3a, b), more in agreement with cells having a relatively high HOXA13 expression showing better survival of the treatment rather than upregulation of expression per se. This was confirmed by analysis of the publicly available single cell RNAseq database recently published by Owen et al.\textsuperscript{21}. Results at single cell level demonstrate the presence of a small population of HOXA13-positive cells in the normal squamous esophagus of BE patients (8%). In BE tissue, the percentage of these HOXA13-positive cells increase to 30%, but their individual HOXA13 mRNA levels are not increased as compared to HOXA13-expressing cells of the normal esophagus (Fig. 3c, d). Similarly, the number of HOXA13-positive cells, but not HOXA13 expression per cell, is increased in IM of the stomach, early gastric cancer, and colorectal cancer (Fig. 3c, d). Thus, we further investigated Hoxa13 at the cellular level in our samples. Although Hoxa13 mRNA expression was detectable in only one of four mice in the upper GI-tract by qPCR (Supplementary Fig. 2), detailed inspection of specimens involved did identify single Hoxa13-positive cells in the stomach of Hoxa13-GFP mice by immunohistochemistry. Such signal was present at the basolateral side along the stomach starting from the GEJ, but not seen in the squamous cells along the esophagus, nor the stroma (Fig. 4a and Supplementary Fig. 7). This is of particular interest as the GEJ has been suggested as a place of origin of BE\textsuperscript{17}. A littermate negative for Hoxa13-GFP showed no positivity (Supplementary Fig. 6d, Supplementary Fig. 7d). Subsequently, we employed ISH for Hoxa13 on surgical samples from the human GEJ of three adult patients to analyze the presence of Hoxa13-expressing cells in the human upper GI tract. In all three specimens, the GEJ junction area contained a clear positive signal for Hoxa13 mRNA, some signal was seen in cells of the proximal stomach, while signal was even lower in the squamous epithelium and stroma (Fig. 4b and Supplementary Fig. 8a, b). Esophageal submucosal glands (ESMG)\textsuperscript{21} were present in one sample and were Hoxa13 positive (Fig. 4c). Interestingly, ESMG were also highly positive for Krt7\textsuperscript{+} Krt5\textsuperscript{+} TP63\textsuperscript{+} cells (previously postulated as the cell of origin BE origin in GEJ\textsuperscript{22}) although unlike Hoxa13\textsuperscript{+} cells, Krt7\textsuperscript{+} Krt5\textsuperscript{+} TP63\textsuperscript{+} positive cells were not identified in the stomach (Fig. 4d). (Of note, this is showed for one sample and we were unable to assess possible Hoxa13 co-expression with these triple positive cells due to absence of specific Hoxa13 antibodies). We also studied Hoxa13 expression in the GEJ of three spontaneously aborted human fetuses of 17–20 weeks of age, a gestation period characterized by transition of the esophageal epithelium from columnar to a squamous phenotype. We observed high and specific Hoxa13 expression at the gastric cardia, while more distal stomach and esophageal epithelium were less positive (Fig. 4e, Supplementary Fig. 8c, d). These data imply that Hoxa13-positive cells are present in the human embryonic
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esophagus during the epithelial transition period, reduced in adult squamous esophagus, and increase again in BE. Thus, the epithelium of both the human and mouse adult upper GI tract, in particular the GEJ and ESMGs for human, is characterized by the presence of a subpopulation of HOXA13/Hoxa13-positive cells in an otherwise HOXA13/Hoxa13-negative surrounding.

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Supplementary Table 1). Using ingenuity pathway analysis (IPA) to further analyze differently expressed genes, a positive association was found with “differentiation of embryonic cells” ($z = 1.82$, $p = 6.38 \times 10^{-15}$). Intriguingly, when HOXA13 expression was induced, cells differentiated less effectively towards definitive endoderm as determined by CXCR4+/E-cadherin+ expression and morphological assessment (Fig. 5c, d). Consistent with a reduced unilinear differentiation, clones expressing HOXA13 showed greater expansion (Fig. 5d).

We next contrasted the transcriptome of non-differentiated, pluripotent HOXA13-overexpressing and control cultures to identify potential molecular mediators of the HOXA13 effects observed. Results of IPA analysis of differential gene expression are broadly consistent with HOXA13 conferring a pluripotent phenotype. Specifically, forced HOXA13 expression results in upregulation of the “role of Nanog in mammalian embryonic cell pluripotency” category ($z = 1.34$, $p = 2.32 \times 10^{-3}$), an effect that involves Sox2, Nanog, Tbx3, Hex-1, and Dppa1 amongst others.27,28 (See Table 1 for more details/results, fold changes, and q-values with regard to this experiment). HOXA13 expression also appears to downregulate Wnt signaling, possibly through BMP signaling29. Wnt signaling is known to promote mesendodermal differentiation30; these results are consistent with HOXA13-mediated downregulation of Wnt signaling during axial elongation31. Thus, the transcriptional profile provoked by HOXA13 is consistent with maintaining a relatively pluripotent phenotype which in turn may increase compartment expansion.

HOXA13 expression does not block endodermal differentiation of mESC cells completely, suggesting that a role for HOXA13 in this compartment is still relevant. Definitive endoderm is a feature of the entire GI tract epithelium, and does not distinguish upper and lower GI epithelium per se. To investigate the role of HOXA13 in this cell compartment and test our prediction that HOXA13 expression would predispose endoderm to acquire distal phenotypes, we sorted CXCR4+ cells only. Healthy, BE Barrett’s esophagus, NAG non-atrophic gastritis, CAG chronic atrophic gastritis, IM intestinal metaplasia of stomach, GC early gastric cancer, and CRC colorectal cancer. $n = 37$ for H, $n = 132$ for BE, NA for NAG, $n = 5$ for CAG, $n = 163$ for IM, $n = 69$ for GC, $n = 37$ for healthy colon, $n = 87$ for CRC of single cells from the individuals mentioned in (c). For GC, statistics are not presented as data per patient was not provided. Boxplots with middle line is the median, the lower and upper hinges correspond to 25th to 75th percentiles, and whiskers representing min-max values.

Fig. 3 Number of HOXA13+ cells rather than cellular expression levels are associated with metaplasia. Exposure to bile (at pH 7) (a) or to acid (b), in two in vitro models of gastroesophageal reflux disease (GERD), marginally induces the expression of HOXA13 from low baseline expression levels in two primary immortalized squamous epithelial cell lines. Error bars represent the 95% CI of the mean. $n = 4$ independent experiments. For HET1a, two-tailed $t$-test was used ($p = 0.0096$ in a, $p = 0.0322$ in b), for EPC2-hTERT Dunn’s multiple comparisons test in a (control vs. 200 µM, $p > 0.99$; control vs. 400 µM, $p = 0.0112$; 200 µM vs. 400 µM, $p = 0.14$), one-tailed $t$-test in b, $p = 0.0486$. c The number of HOXA13-positive cells is increased in Barrett’s esophagus (BE) and intestinal metaplasia (IM) as compared to normal esophagus or stomach tissue. Healthy and BE esophageal samples are derived from the same patients. Mean ± SEM are shown. $n = 4$ individuals for esophagus, $n = 3$ for NAG, CAG, $n = 4$ for IM, $n = 1$ for GC, $n = 6$ for healthy colon, $n = 11$ for CRC. Graphs are based on the analysis of single cell RNA data seq21, GSE13452032, GSE81683. Two-tailed $t$-test was used for esophagus ($p = 0.0443$) and colon ($p = 0.6808$), one-way analysis of variance for stomach with Dunnett’s Multiple Comparison Test ($p < 0.0001$). d HOXA13 expression level per cell is unchanged. Expression level presented in HOXA13+ cells only. H healthy, BE Barrett’s esophagus, NAG non-atrophic gastritis, CAG chronic atrophic gastritis, IM intestinal metaplasia of stomach, GC early gastric cancer, and CRC colorectal cancer. $n = 37$ for H, $n = 132$ for BE, NA for NAG, $n = 5$ for CAG, $n = 163$ for IM, $n = 69$ for GC, $n = 37$ for healthy colon, $n = 87$ for CRC of single cells from the individuals mentioned in (c). For GC, statistics are not presented as data per patient was not provided. Boxplots with middle line is the median, the lower and upper hinges correspond to 25th to 75th percentiles, and whiskers representing min-max values.

Fig. 4 Additional relevant molecules. A significant increase in expression of a large number of molecules is observed which may play a role in the development of Barrett’s esophagus. Gene expression is shown separately for the in vitro model systems of GERD (a), and for the in vivo model systems of GERD in duodenal tissue (b). Results of IPA analysis of differential gene expression show that a large number of molecules are upregulated in Barrett’s esophagus (BE) and intestinal metaplasia (IM) as compared to normal esophagus or stomach tissue. Healthy and BE esophageal samples are derived from the same patients. Mean ± SEM are shown. $n = 4$ individuals for esophagus, $n = 3$ for NAG, CAG, $n = 4$ for IM, $n = 1$ for GC, $n = 6$ for healthy colon, $n = 11$ for CRC. Graphs are based on the analysis of single cell RNA data seq21, GSE13452032, GSE81683. Two-tailed $t$-test was used for esophagus ($p = 0.0443$) and colon ($p = 0.6808$), one-way analysis of variance for stomach with Dunnett’s Multiple Comparison Test ($p < 0.0001$). d HOXA13 expression level per cell is unchanged. Expression level presented in HOXA13+ cells only. H healthy, BE Barrett’s esophagus, NAG non-atrophic gastritis, CAG chronic atrophic gastritis, IM intestinal metaplasia of stomach, GC early gastric cancer, and CRC colorectal cancer. $n = 37$ for H, $n = 132$ for BE, NA for NAG, $n = 5$ for CAG, $n = 163$ for IM, $n = 69$ for GC, $n = 37$ for healthy colon, $n = 87$ for CRC of single cells from the individuals mentioned in (c). For GC, statistics are not presented as data per patient was not provided. Boxplots with middle line is the median, the lower and upper hinges correspond to 25th to 75th percentiles, and whiskers representing min-max values.
**HOXA13 and the chromosome 1 epidermal differentiation complex.** Further support for a role of **HOXA13** in the loss of the squamous phenotype and the appearance of caudal columnar phenotypes in the esophagus comes from experiments in which we investigated the effect of **HOXA13** directly on esophageal cell models. To this end, we used CRISPR-Cas9 technology to delete **HOXA13** from BAR-T, a primary monoclonal immortalized cell line derived from metaplastic tissue of a BE patient, with cells expressing both columnar and squamous markers. Three separate **HOXA13** knock-out clones were selected to circumvent potential off target effects. Reversely, we provoked lentivirus-mediated **HOXA13** expression in EPC2-hTERT, an immortalized squamous esophageal cell line. For these latter experiments we used a mixed cell population of lentivirally transduced cells as to avoid clonal artifacts influencing results. Transcriptomes in these two models (Fig. 6a) were contrasted to their respective control lines. There was substantial overlap in the gene sets significantly affected by losing **HOXA13** in BAR-T compared with those significantly affected by gaining **HOXA13** in EPC2-hTERT, taking into account the direction of regulation (X2 test: \( p = 4.74 \times 10^{-34} \)) (see Supplementary Data 3). Investigation of this overlap across the two technically independently generated datasets limits the incidence of chance findings or single model system bias. Overlapping genes positively affected by **HOXA13** expression in esophageal cells are **IL7r**, **FAM196B**, **ADAMTS6**, **NRG1**, **LTBP1**, **JAG1**, **ELL2**, **SMAD7**, **C12ORF75**, **AXL**, **TIPARP**, **IKBIP**, **DUSP7**, and **GOLIM4**. Down-regulated by **HOXA13** expression are **SERPINB13**, **MYO5C**, **KLK7**, **ANXA9**, **TMPRSS4**, **TCC9**, **MATN2**, **TNFAIP2**, **RAB27B**, **HCAR2**, **C6ORF132**, **EXPH5**, **MAP3K5**, and **FUCA1**. IPA analysis of the results predicts an increase in “(malignant) cell transformation” (\( z = 2.00, p = 5.81 \times 10^{-3} \)) and a decrease in “inflammation of an organ” (\( z = -2.59, p = 8.29 \times 10^{-3} \); gene function is described in Fig. 4 **HOXA13** expression in the upper GI tract. **a** Representative example of anti-GFP immunohistochemistry of a Hoxa13-GFP heterozygous mouse with gastroesophageal junctions (GEJ) (SQ; squamous epithelium, ST; stomach) (n = 3). Hoxa13 is expressed in single cells of the stomach starting from the GEJ (5–6) and absent in the esophagus and stroma (1–4). **b** **HOXA13** expression as measured by RNA ISH in a representative example from n = 3 with similar results of an adult human GEJ with magnification panel of: A—esophagus, B—GEJ area, C—proximal stomach. Orange circles indicate the positive signal in the overview image. **c** **HOXA13** expression as measured by RNA ISH in human esophageal submucosal gland (ESMG) with magnification panel of: A—H&E, C,D—ESMG, B,E—squamous esophagus, n = 1. **d** Keratin 7 (KRT7), keratin 5 (KRT5) and p63 triple positive cells are found in the ESMG, n = 1. **e** Overview of a representative example of a 17-week old fetus GEJ: A) Stratified esophageal epithelium of the distal esophagus (blue), B) GEJ area, C) gastric epithelium of the proximal stomach (pink), n = 3.
Fig. 5 HOXA13 cellular expression modulates cell fate. a HOXA13+ cells of normal esophagus cluster together with BE cells in t-distributed Stochastic Neighbor Embedding (T-SNE) plot based on single cell RNA expression profiling21. b Analysis of single cell RNA seq data revealed that in contrast to HOXA13− cells, HOXA13+ cells express submucosal gland markers, Barrett’s esophagus (BE) markers and have decreased expression of squamous markers (p63, IVL) in healthy esophagus. This difference is not observed in BE. n = 846 of HOXA13− cells in healthy esophagus, n = 37 of HOXA13+ in healthy esophagus, n = 263 HOXA13− cells in BE, n = 132 of HOXA13+ cells in BE. c HOXA13-overexpressing definitive endoderm is relatively resistant to terminal differentiation. Mouse embryonic stem cells (mESC) cells with and without forced HOXA13 expression were differentiated from pluripotent stem cells to definitive endoderm. The percentage of differentiated definitive endoderm cells, defined as CXCR4+/E-cadherin+ cells, was analyzed by FACS analysis (upper panels). Lower panels (representative light microscopy images) show morphological differences in cultures of HOXA13 overexpressing and wildtype mESCs upon differentiation to definitive endoderm, which induces a flattening of cell layers, with larger and irregular shaped cells. d Quantification of FACS analysis results indicates that the percentage of CXCR4+/E-cadherin+ cells is decreased in HOXA13-overexpressing cell cultures under differentiation conditions (p < 0.0001). HOXA13-expressing cells expand faster during the differentiation process compared to control cells (total number of cells increased) (p = 0.0135). Mean ± SEM, ***p < 0.001, n = 4 independent experiments, t-test (two-tailed). e Model of cellular identity in BE development. The X-axis represents time (hypothetical units) following exposure to GERD-inducing agents. Y-axis shows differentiation during embryology and pathology. Z-axis indicates the positional identity of GI-tract tissues. Several theories exist regarding the cell of origin of BE: they may be fully differentiated esophageal or stomach cells, or less differentiated cells within these organs (depicted by the 4 cells on the Y–Z plane). Irrespective of its location or differentiation state, this cell or origin might lose its correct positional identity or maintain its aberrant positional identity and resembles a definitive endoderm like cell. This is visualized by the blue rectangle harboring the cell with the thicker blue contour. For the model of cellular identity in BE, our data suggest that HOXA13 expressing clones in the GEJ, depicted in orange, may outcompete clones with another positional identity, providing an explanation for the distal phenotype observed in BE.
Supplementary Data 3) in cells expressing HOXA13. Intriguingly, HOXA13 downregulates the epidermal differentiation complex (EDC); Fig. 6b, c). The EDC, located on chromosome 1q21.3, contains clustered multigene families of genes associated with cornified envelope formation in stratified squamous epithelia, such as the S100 and the small proline-rich region (SPRR) genes. Among the overlapping downregulated genes in both cell models, ANXA9 is also associated with differentiating keratinocytes, and EVPL, SEL, and KLK7 are cornified envelope genes. EMP1 and SERPINB13 downregulation is associated with increased disease severity in gastric cancer (EMP1) and head and neck squamous cell carcinoma (SCC) (SERPINB13). See Table 1 for fold changes and q-values and Supplementary Data 3 for more differentially expressed molecules related to morphology. The downregulation of a gene region known to be essential for maintaining a squamous phenotype provides mechanistic support to the notion that altered HOXA13 expression is cardinal for provoking the BE phenotype.

These experiments also provide mechanistic support for the notion that HOXA13 expression may offer an explanation as to why BE is prone to progression to EAC. HOXA13 mediates down-regulation of the EDC and many EDC and cornified envelope genes are progressively down-regulated in the BE to EAC cascade.

In BE, EAC, and esophageal SCC, loss of heterozygosity (LOH) of the EDC is common. Low EDC gene expression predicts chemotherapy non-response and LOH of the EDC is associated with reduced survival in curatively treated EAC patients.

In our experimental models, we observed HOXA13-mediated upregulation of genes associated with Notch signaling, specifically DLL1, FURIN, and JAG1. Notch signaling is associated with malignant transformation.

In IPA analysis "Non-melanoma solid tumor" (z = 2.03, p = 9.28 × 10−8) and "invasion of cells" (z = 2.08, p = 2.47 × 10−3) were shown to be activated by HOXA13, whereas HOXA13 expression negatively influenced the "Apoptosis" (z = −1.52, p = 2.23 × 10−3) and "killing of cells" (z = −2.03, p = 2.03 × 10−3) categories. Many individual genes showed differential regulation in a pro-oncogenic direction (see Supplementary Data 3).

In conclusion, using HOXA13 knock-out and overexpression in a Barrett’s and a squamous cell line, we show that HOXA13 downregulates the epidermal differentiation complex and other cornified envelope genes which normally function to maintain squamous epithelial morphology and act as tumor suppressor genes. Additionally, Notch signaling is overexpressed and many individual genes show differential regulation in a pro-oncogenic direction.

Table 1 Fold changes and q-values for the mRNAs mentioned in the results section of the main text pertaining to cell culture models analyzed by RNA-Seq.

| Gene name | Fold change | q-value (multiple testing corrected p value) |
|-----------|-------------|--------------------------------------------|
| Forced HOXA13 in mESC confers a relative competitive advantage in multipotent cell cultures through upregulation of Nanog signaling and downregulation of Wnt signaling | Sox2 | 1.43 | 0.01 |
| Nanog | 1.47 | 0.00 |
| Tbx3 | 3.53 | 0.00 |
| Hexx1 | 20.56 | 0.00 |
| Dppa1 | 64.95 | 0.00 |
| Igf2 | 3.61 | 0.00 |
| Wnt3 | 0.43 | 0.00 |
| Wnt4 | 0.36 | 0.00 |
| Wnt6 | 0.36 | 0.00 |
| Wnt8a | 0.48 | 0.01 |
| Sp8 | 0.12 | 0.00 |
| Lef1 | 0.41 | 0.00 |
| Tbx3 | 0.41 | 0.00 |
| Axin2 | 0.55 | 0.04 |
| Fgfl | 0.10 | 0.00 |
| Cdx1 | 2.47 | 0.00 |
| Grl3 | 5.00 | 0.00 |
| Vill | 1.87 | 0.00 |

- Forced HOXA13 expression supports caulial epithelial functions and appears to promote proliferation in DE
- Sox17: 12.55, q = 0.00
- Lgr5: 5.39, q = 0.00
- Nanog: 0.20, q = 0.02
- Ezh: 2.20, q = 0.00
- Vill: 2.58, q = 0.048
- Krt19: 2.34, q = 0.00
- Krt20: 2.84, q = 0.01
- Igf8: 4.67, q = 0.00
- Gcnt3: 3.51, q = 0.00
- Fgf2: 2.81, q = 0.00
- Nek2: 2.35, q = 0.01

- HOXA13 downregulates the chromosome 1 epidermal differentiation complex, is pro-oncogenic, and conveys typical characteristics of the BE phenotype
- ANXA9: 0.48, q = 0.04
- EVPL: 0.61, q = 0.03
- SEL: 0.52, q = 0.01
- KLK7: 0.42, q = 0.01
- EMP1: 0.56, q = 0.03
- SERPINB13: 0.38, q = 0.00
- DLL1: 2.57, q = 0.00
- FURIN: 1.49, q = 0.02
- JAG1: 1.85, q = 0.04

HOXA13 supports columnar phenotype and provides proliferative advantage. Having established the transcriptional effect of HOXA13 on BAR-T and EPC2-hTERT cells, we next investigated the functional consequences of HOXA13 in these cells. As was seen for mESCs, HOXA13 expression significantly enhances the growth-rate of esophageal cells. For BAR-T cells, a proliferative advantage of HOXA13 expression was seen in 2D cultures (Fig. 6d), while for EPC2-hTERT the positive effect of HOXA13 expression on cell growth was more noticeable under 3D culture conditions (Fig. 6e). Moreover, HOXA13 expression decreases the sensitivity of keratinocytes to bile/acid exposure (Fig. 6f), consistent with the notion that HOXA13 confers cellular protection under GERD-like conditions.

To gain further insight into the role of HOXA13 in cell morphology and organization, we made use of the fact that EPC2-hTERT cells can be differentiated in 3D spheroid cultures, and become organized in layers with a more flattened cytological aspect in the middle of spheroids and high expression of keratinization markers such as involucrin, similar to esophageal stratified epithelium (see example in Fig. 7a top panel for differentiated morphology). Upon overexpression of HOXA13, EPC2-hTERT spheroids increase in size while maintaining a less differentiated phenotype (undifferentiated morphology, Fig. 7a bottom panel). Quantification of these morphological states indicates that in control cultures, 80% of spheroids attain a stratified epithelial phenotype, while overexpression of HOXA13 reduces this number to 28.6% (p < 0.05, Fig. 7b left panel). This was further confirmed by staining for involucrin as a marker of keratinization, showing a decreased expression in spheroids derived from HOXA13-overexpressing EPC-hTERT cells (p < 0.05, Fig. 7b right panel). Thus, in primary immortalized esophageal cells HOXA13 overexpression reduces keratinization.
We further investigated the morphological role of HOXA13 in the BE-derived BAR-T cell line. In 2D cultures, an altered spatial distribution in growth pattern was observed, with cells growing more closely together in the absence of HOXA13 suggesting an effect on tissue morphology (Fig. 7c). The BAR-T cell model also allows testing the effect of HOXA13 on columnar versus squamous differentiation in in vitro and in vivo settings. A 3D in vivo tissue reconstitution model was employed in which BAR-T cells were grafted in the lumina of devitalized and denuded rat tracheas and implanted in NOD SCID mice. Under these conditions, parental non-transfected BAR-T cells produce both intestinal-type columnar epithelium and stratified squamous epithelium from the same clone. Hence this cell line has the potential to produce two types of morphological distinct epithelia35,49 (Fig. 7e and Supplementary Fig. 9). Thus the epithelium in the model finds itself on a tipping point between both morphologies. This characteristic makes the in vivo tissue reconstitution model suited for studying the influence of
modulators of morphology, i.e. to show if the modulator favours intestinal-type columnar epithelium or stratified squamous epithelium. Studying the effect of HOXA13 knock-out, two important observations were made. Firstly, HOXA13 knock-out decreases the length of columnar-like epithelium which contains PAS positive cells and is negative for involucrin (Fig. 7e, f). Thus, loss of HOXA13 counteracts the proliferation of the intestinal-type columnar epithelium while the stratified squamous epithelial proliferation remains present. Secondly, HOXA13 knock-out impairs epithelial proliferation in general, as inferred from the thickness of the epithelial layer (Fig. 7d; Supplementary Fig. 9b). In vitro 2D organotypic ALI cultures of these cell lines confirm the in vivo findings, with HOXA13 knockout reprogramming the BAR-T epithelial cells towards a squamous keratinized differentiated epithium (Fig. 7f). In conclusion, HOXA13 supports intestinal-type columnar epithelial differentiation and proliferation of the Barrett’s epithelium confirming the notion that HOXA13 expression can mediate both a competitive advantage as well as a predisposition to the formation of columnar phenotypes.

Discussion

In this study, we characterized HOX gene expression and localization in mice and men, demonstrating a collinearity of these genes along the GI tract. Following analysis of one of these HOX genes, HOXA13, we observed single HOXA13+ cells in the upper GI tract, which present exceptions to the HOX gene collinearity theory. Specifically, in the normal physiology of the esophagus and proximal stomach, non-squamous structures such as the epithelium at the GEJ, glandular cells of ESMGs and glands of stomach contain single cells expressing HOXA13. The fact that these cells have not been described before may be a reflection of the fact that homogenization of tissues for qPCR masks this fraction, and that single cell analysis of the GI tract for this gene has not been performed before. We observe that GI pathology with distal phenotypes like intestinal metaplasia of the esophagus and stomach are characterized by an expansion of HOXA13-positive cells, while conversely, a relatively low expression of HOXA13 is found in the phenotypically rostral Paneth cell metaplasia and pyloric metaplasia of the colon, compared to the surrounding physiological tissue.

It is clear that in normal physiology, HOXA13 contributes to the distal phenotype of the caudal GI tract, beginning the question as to the role and origin of the HOXA13-expressing compartment now observed in the upper GI tract. We demonstrate that esophageal HOXA13-positive cells express columnar and BE markers and show gene expression patterns overlapping with BE-derived cells. Functionally, HOXA13 provides cells with several properties required for development of a BE segment. HOXA13 maintains cells in a stem-like progenitor state, while conferring a proliferative advantage, promoting cellular migration and resistance to bile and acid exposure. Furthermore, in cells that are lineage committed, HOXA13 supports a phenotypically columnar phenotype, most likely partly driven by downregulation of the chromosome 1 epidermal differentiation complex. Thus, our data are consistent with the hypothesis that BE arises as a consequence of the expansion of resident HOXA13-positive cells under abrasive environments such as GERD. Several potential theories have been proposed as to the origin of BE: transdifferentiation of basal cells in the squamous epithelium, extension of a special population of cells from the GEJ, repopulation of the esophagus after injury with cells derived from progenitors ESMGs or ducts, resident embryonic stem cells or circulating bone marrow cells. These potential sources of esophageal columnar epithelium are not mutually exclusive, and BE may have more than one precursor cell or location. Our study supports the previously proposed hypothesis that BE may originate from ESMGs and the GEJ as HOXA13 is expressed in OLM4+, LEFTY1+ cells of ESMGs, recently suggested as a cell of BE origin. The fact that in addition to the GEJ, rare HOXA13+ cells are found in the human esophagus and stomach, is consistent with the observation that after esophagogastrectomy BE can reoccur in patients, indicating that the involvement of the GEJ is not an absolute prerequisite for the development of BE. Furthermore, our data show that HOXA13 is already present at stem cell level, supportive of the notion that BE may arise from a cell with stem-cell like characteristics. While HOXA13 expression overlaps greatly with KRT7, a columnar cytokeratin seen in Barrett’s, we did not observe direct transcriptional overlap with the previously described KRT7+KRT14/S5+TP63+ cell of BE origin. However, KRT7+KRT5+TP63+ cells gave rise to BE-like epithelium only upon ectopic expression of CDX2. Lineage-tracing studies are needed to further confirm whether one or more types of cells of origin might exist for BE. While we focused on HOXA13 here, it is conceivable that other HOX paralogues are involved in BE pathophysiology, in particular caudal genes such as HOXA10, 11, 13, and 10 are interesting candidates for further investigation, in particular as disruption of collinearity was reported for cluster B in BE and in duodenum of murine embryos.

BE is considered as the precursor lesion for EAC, a dangerous form of cancer of which the incidence has substantially increased in recent decades. Increased insight into the pathogenesis of BE may aid development of prevention and treatment strategies for EAC. HOXA13 is involved in ESCC and other types of cancer. Here we show that expression of HOXA13 also increases in EAC and colorectal cancer, provides proliferative advantage to the cells and activates cancer-related gene transcription like Notch signaling. Hence, we speculate that HOXA13 may play a role in BE progression towards EAC.

In toto, the present study identifies a importance of regional patterning by HOX genes in the gut epithelium. In Barrett’s esophagus, gastric IM, and heterotopia of the upper GI-tract, a colon-like HOX gene expression is present, especially
characterized by HOXA13 upregulation. Single cells expressing the generally thought to be distally-restricted HOXA13 gene are present in the physiological upper GI tract, in particular the GEJ, where it supports a columnar phenotype and may confer a relative competitive advantage. Thus, HOXA13 mediates BE phenotype and proliferative potential and hence appears a rational target for strategies aimed at counteracting EAC development.

Methods
Collection of human material. All human tissues used in this study were obtained at the Erasmus University Medical Center, department of Gastroenterology & Hepatology. The use of these samples was approved by the Erasmus MC medical ethical committee (MEC-2015-208, MEC-2015-209, MEC-2015-199, MEC-2010-093; tissues were handled according to the FEDERA code of conduct and informed consent was obtained for all participants58. The study was designed and carried out according to the ethical principles for medical research involving human
subjects from the World Medical Association Declaration of Helsinki. Biopsy specimens to investigate HOX collinearity were obtained by double balloon enteroscopy. Nine biopsy specimens were obtained from each patient (n = 3) at different locations along the GI tract. Sequentially these locations were: esophagus, stomach, duodenum, jejunum, proximal ileum, distal ileum, ascending colon, descending colon, and rectum. (Supplementary Fig. 2). Included patients had unexplained symptoms, mostly anemia, while inflammatory bowel disease patients were excluded. All biopsies for RNA isolation were stored in RNA-later at −80 °C. Squamous esophageal biopsies (n = 13) originated 5 cm above the squamocolumnar junction (SCJ). Barrett’s (BE) biopsies (n = 13) originated caudal of the SCJ and cranial of the gastric folds (all patients were on PPI therapy), stomach biopsies (n = 12) were from the corpus. All three types of biopsy specimens were derived from the same patients, (one stomach biopsy specimen was not obtained due to patient agitation during the gastroscopy). The squamous esophageal, BE, and stomach biopsies were taken in a paired fashion from 13 patients. Where the number of samples is indicated below, this indicates the number of individual patients. Forceps biopsy specimens of EACs (n = 12) were obtained. Pathological examination of simultaneously taken forceps biopsies around the study specimens had to be positive for EAC. Gastric inlet patch were sampled from proximal esophagus. To determine the proximal colonic HOXA13 border, biopsies were taken from the cecum at the appendix base, the ileocecal valve, 5 cm distal to the ileocecal valve, and from the transverse colon in each patient (n = 5).

Collection of archival pathology specimens. FFPE material was collected from gastric IM (from the antrum, angularis, and corpus, i.e., not from the cardia; n = 12), the gastric inlet patch (n = 5), CLE (from the proximal esophagus; n = 14), and Meckel’s diverticula (n = 14). For RNAscope RNA-Ish, one FFPE specimen of each of these origins was used. Depending on the extent of metaplasia, remainder of tissues was used whole, macroscopically separated, or processed with the Photo-Activated Localization Microscopy with the Laser Caption Microdissection (PALM LCM) for mRNA isolation and subsequent qPCR. Nuclease-free membrane slides were used to collect the tissue of interest after cutting and pulsing of the PALM LCM. Additionally, FFPE materials or fresh pinch biopsies were collected from the same patients, (one stomach biopsy specimen was not obtained due to patient agitation during the gastroscopy). The squamous esophageal, BE, and stomach biopsies were taken in a paired fashion from 13 patients. Where the number of samples is indicated below, this indicates the number of individual patients. Forceps biopsy specimens of EACs (n = 12) were obtained. Pathological examination of simultaneously taken forceps biopsies around the study specimens had to be positive for EAC. Gastric inlet patch were sampled from proximal esophagus. To determine the proximal colonic HOXA13 border, biopsies were taken from the cecum at the appendix base, the ileocecal valve, 5 cm distal to the ileocecal valve, and from the transverse colon in each patient (n = 5).

**Fig. 7** HOXA13 supports intestinal-type columnar epithelial differentiation. HOXA13 overexpression impairs squamous differentiation of EPC2-hTERT spheroids as seen from the representative pictures (a) and quantitative assessment of morphologies based on hematoxylin and eosin (H&E) or anti-involucrin (IVL) immunohistochemistry (IHC) (p = 0.0086). (b) Median with interquartile range, *p < 0.05, Mann–Whitney test (two-tailed). HOXA13 knockdown (KO) affects spatial distribution of BAR-T cells. Mean ± SEM, **p < 0.001, t-test (two-tailed). (c) The length of columnar and mixed BAR-T epithelium decreases upon HOXA13 KO in the rat trachea in vivo tissue reconstitution model. Mean ± SEM, **p < 0.05, exact p = 0.0439.

d Representative examples of H&E, PAS staining, anti-IVL IHC of BAR-T epithelium from the rat trachea in vivo tissue reconstitution model. H&E staining shows more layers of cells in animals transplanted with HOXA13+/− wild type cells. Periodic acid–Schiff (PAS) stains polysaccharide molecules, and positivity is indicative of goblet-like cells. The arrows point to PAS positivity, which is present in the right panel but not in the left panel, where the BAR-T HOXA13− cells are shown. IVL staining is strong in morphologically squamous cells in the left hand panel and weaker in the HOXA13+/− epithelium (n = 3 for HOXA− and n = 5 for HOXA+). HOXA13− and HOXA13+ representative pictures of H&E staining, PAS staining and IVL IHC of the BAR-T organotypic cell culture system indicate that HOXA13 KO reprograms the columnar epithelial phenotype towards squamous keratinized epithelium (n = 1 independent experiments).

**Immunohistochemistry.** For immunohistochemistry, slides were blocked in 10% of normal goat serum, antigens were retrieved by boiling samples in citrate buffer (pH6), and samples were incubated overnight at 4 °C with primary antibody. Dilation and manufactures of primary antibodies are presented in the Supplementary Table 4. After incubation with HRP-conjugated secondary antibody (Dako EnVision+System-HRabbit labeled Polymer Anti Mouse, Dako) endogenous peroxidase was blocked in 3% H2O2 and antibody binding was visualized by DAB staining. IHC analysis for HOXA13 was tried using antibodies ab106503 and ab26084, however these failed to show specificity and have since been discontinued by the companies offering them. H&E staining was performed by 60. For H&E stainings de-paraffinized 4 µm slides were incubated during 3 min in hematoxylin solution, followed by tap water washes and 15 s of incubation with eosin. For PAS staining, de-paraffinized slides were incubated with 0.5% Periodic Acid solution for 10 min, followed by two ddH2O washes and incubation in Schiff’s reagent (Sigma Aldrich) for 15 min and hematoxylin for 3 mins.

**Multiplex immunofluorescent staining.** Triplest staining for keratin 5, keratin7 and p63 was done by automated multiplex IF using the Ventana Benchmark Discovery (Ventana Medical Systems Inc.). In brief, following deparaffinization and heat-induced antigen retrieval with CCI (950–500, Ventana) for 64 min at 97 °C, the tissue samples were incubated firstly with Keratin 5 antibodies for 32 min at 37 °C followed by detection with Ultramap anti-rabbit HRP (7670–4315, Ventana) for 12 min followed by visualization with Red610 for 8 min (7670–245, Ventana). Antibody denaturing was performed using CC2 (950–123, Ventana) for 20 min at 100 °C. Secondly, Keratin 7 antibodies were incubated for 32 min at 37 °C followed by detection with Ultramap anti-rabbit HRP (7670–4315, Ventana) for 12 min followed by visualization with Cy5 for 12 min (7670–238, Ventana). Slides were incubated in PBS with DAPI for 15 min and covered with anti-fading medium (DAKO, S3023).

**RNA isolation.** RNA was isolated using the NucleoSpin RNA isolation kit (Macherey Nagel, Düren, Germany). Biopsies and animal tissues were homogenized by the TissueRuptor obtained from Qiagen Inc. RNA concentrations were measured using a Nanodrop spectrophotometer and samples were stored in RNA storage solution (Sodium Citrate pH 6.4), bought from Ambion (Foster City, USA) and kept at −80 °C. RNA integrity was checked with 1% agarose gel-electrophoresis. FFPE material was deparaffinized with xylene and ethanol, lysed, digested with proteinase K, and RNA was isolated with the High Pure FFPE RNA isolation kit obtained from Roche (Basel, Switzerland). RNA isolation from de-differentiated KH2 mouse embryonic stem cells (mESCs) was done using a picopure RNA isolation kit (Thermo-Fisher Scientific, Waltham, USA). After RNA isolation all samples for RNA-Sequencing were tested on the Agilent 2100 Bioanalyzer to determine RNA integrity and quality.

**RNA expression is driven by the endogenous mouse *Hoxa13* promoter through the creation of a fusion protein59.** Mice were generally kept with 12:12 h light–dark, the animal room temperature is between 20 and 24°C and the relative humidity is 55 ± 10%. These tissues were taken out and embedded in O.C.T. Compound bought from Quagen Inc. (Hilden, Germany) and frozen at −80°C. Cryosections were made which were mounted in fluoroshield mounting medium with DAPI obtained from Abcam (Cambridge, UK). Subsequently, the GE and the distal GI-tract were analyzed directly for GFP expression using the Zeiss confocal laser scan microscope LSM 510. Additionally, immunohistochemistry staining was performed with anti-GFP antibody (#AB3080, Bio-Connect BV) (see below). These murine experiments were approved by the Ethical Committee for Animal Experiments of the Erasmus MC and were performed according to the guidelines of the same institution.

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and qPCR. cDNA was made from 1 µg RNA using Primerscript RT Master Mix according to manufacturer’s instructions (Takara, Otsu, Japan), for 15 min at 37 °C. 5 µl of cDNA was then PCR-amplified at – 95 °C for 1 min, followed by 40 cycles in the iQ5 Real-Time PCR detection system that was obtained from BioRad Laboratories (Veenendaal, The Netherlands). For each reaction 10 µl cDNA template, 12.5 µl SYBR GreenER purchased from Invitrogen (Carlsbad, CA), and 2.5 µl 10 pM/µl primer were used. Reactions were performed in duplicate. Primers used are shown in Table 2 and were ordered at Sigma-Aldrich (St. Louis, Staat, Germany). qPCR data were analyzed with Microsoft Excel using the ΔΔCt method. Reference genes used for PCR experiments on human materials were β2-M, β-ACTIN, and GAPDH. Reference genes used for PCRs on mice materials were Eef2, RpL37, and 18S. Differences in expression were analyzed with a two sided Student’s t-test using Prism 5.01, obtained from GraphPad Software (San Diego, USA). Values from individual samples were excluded if they deviated more than 2 SD from the mean. Correlations between HOTTIP expression in the squamous esophagus and BE, and correlations between HOTTIP and HOXA13 expression levels in the squamous esophagus and BE were tested using nonparametric Spearman correlations. Representation of all in gographs, i.e. cells mapping higher or lower than 1.5x the median, indicating the paired nature of the specimens, i.e. they are derived from the same patient, used for this analysis.

In situ hybridization by RNAscope. RNAscope was performed according to the instructions of the manufacturer of the probes and the reagent kit (VS Reagent Kit 2.30.0, GEOquery 2.40.0, limma 3.26.863. The results were represented as a fold-change (log2). In Microsoft Excel, this log2 FC was converted to fold change (%). For each log2 FC, an empirical Bayes moderated t-statistic was calculated, p-values were corrected for multiple testing using the Benjamini & Hochberg false discovery rate method.

Analysis of single cell RNA seq datasets. BE and ESMG Single Cell Experiment Matrix from supplementary Data files 6 & 3 Experiment Matrixes have been mapped to hg38 standard human genome (TXDb.Hsapiens.UCSC.hg38-known- Gene R-package), normalized as Reads Per Kilobase per Million mapped reads (RPKM). Genes with an expression level less than in 0.5% of all cellularities were excluded based on: (1) the number of expressed genes—for 10x Single-Cell sequence data, cells expressing less than 400 or more than 7000 genes, for smartSeq data cells expressing less than 1000 and more than 7000 were removed. Different numbers were chosen due to the different sequencing depth. (2) Boxplot representation of all cells—outliers, i.e. cells mapping higher or lower than 1.5x the first or third quartile were removed. (3) Based on % of reads - cells were removed if there were more than 20% of reads mapping to mitochondrial or ribosomal genes. HOXA13-related genes query: HOXA13-positive cells from normal esophagus were selected with R. Genes that were expressed in at least 70% of these HOXA13-positive cells (20445) were analyzed for their expression in HOXA13-negative cells of normal esophagus as well as HOXA13 negative and positive cells in BE tissue. For T-SNE plot, 638 cells were included (388 cells from Barrett’s tissue, 250 cells from normal esophagus) and plotted based on their location of origin (colour) as well as HOXA13 expression (open vs closed symbols).

Cell culture. All cells were cultured with penicillin (100 µg/ml) and streptomycin (100 µg/ml) and were regularly STR-verified and checked for mycoplasma by handing in samples prepared according to instructions at GATC Biotech (Konstanz, Germany). Primary human esophageal epithelial cells transformed with tERT (EPC2-tERT) (gift of K.K. Krishnadath) were cultured with Keratinocyte SFM medium, supplemented with bovine pituitary extract at 50 µg/ml and EGF at 1 ng/ml (Thermo Fisher Scientific). HEITA, the primary immortalized human squamous esophageal cell line Ht-1A was a gift of J.W.P.M. van Baal (University Utrecht, The Netherlands). These cells were grown in EPM2 medium obtained from AthenaES, (Baltimore, Maryland, USA). The primary human BE cell line (BAR-T) was a gift of dr. J.W.P.M. van Baal who had, in turn, received it as a gift from Dr. E. van der Groep (University Utrecht, The Netherlands). These cells were grown in supplemented keratinocyte basal medium (KBM2), bought from Lonza (Basel, Switzerland), according to the method of Jaiswal et al.65. HK2 mESCs were a gift of J. Grimbau and maintained in DMEM with 10% FCS, Non-Essential Amino Acids, sodium pyruvate, LIF, and β-ergocornithine from Lonza (Basel, Switzerland). Embryonic stem cell medium; Supplementary Table 3). Dishes were coated with attachment factor protein solution (Thermo-Fisher Scientific). Irradiated mouse embryonic fibroblasts (3T3-Swiss albino cells (gift of J.W.P.M. van Baal), cultured in DMEM with 10% FCS, were used as feeder cells. HEK293T cells were cultured in DMEM with 10% FCS.

Generation of EPC2-tERT HOXA13 overexpression model. The human HOXA13 gene including its single intron was amplified using Q5 polymerase from cDNA using primers (Agen HOX A3 F: GGTGTTGACCGTGATCCAGA GCTCTCCGGCTGCCCT, and Xbal HOX A3 R: AACCACCTCTAGAATAC AGTTGGTTTTCAGTT) and cloned into pEN_TmiRc3 using AgeI and Xbal restriction sites, a gift from Iain Fraser (Addgene (Cambridge, USA) #25748)66. SLMK-Venus was transferred into the HOXA13 insertion site of the VS Reagent Kit instructions (Takara, Otsu, Japan), for 15 min at 37 °C and 5 s at 95 °C, and stored at 4 °C. Two cloning experiments were performed: one for the generation of a Gateway reaction70. SLMK-Venus was a gift from Iain Fraser (Addgene #25734)66. A similar plasmid but without the HOXA13 insert served as control. Both plasmids were sequenced by LGG Genomics (Teddington, UK). Next, plasmids were packaged into lentiviral particles following transfection in HEK293T cells with third-generation packaging plasmids. The supernatant was collected and ultracentrifuged. EPC2-tERT cells were transduced with the virus and Fluorescence-Activated Cell Sorted (FACS) for YFP (pSLK-Venus) positive cells on the BD FACSCountTM II that was bought from BD Biosciences (San Jose, USA). These cells were grown and analyzed as a cell pool. HOXA13 was induced by the addition of 0.05 µg/ml doxycycline to the culture medium. Overexpression was determined by qPCR according to scientific standards66.

Generation of HK2 embryonic stem cells HOXA13 overexpression model. The human HOXA13 gene including its single intron was amplified using Q5 polymerase from cDNA using primers with an added N-terminal FLAG-tag sequence (GACTACAAGACAGATGACGACAAAG) and Kozak sequence (GGCCGCCAC; Supplementary Table 3). Next, the plasmid was digested using EcoRI digested pGK-ATG-frt (Addgene #20734) using Gibson Cloning (New England Biolabs Inc., Ipswich, MA). pGK-ATG-frt was a gift from Rudolf Jaenisch66. HK2 mESCs were passaged the day before the electroporation and four hours before electroporation medium was replaced. Approximately 1.5-10² HK2 cells were electroporated with 50 µg of pGK-ATG-frt-HOXA13 and 25 µg of pPCGGS-FLPe-puro (Addgene #20733)66. Cells were electroporated in 4 mm cuvettes, with two consecutive pulses (400 V/250 µF) using a Gene PulserXcell (Bio-Rad Laboratories). The next day 140 µg/ml Hygromycin B (Thermo-Fisher Scientific) was added for the selection of correctly targeted colonies. DNA from resistant colonies was isolated with the Kleargene XL blood DNA extraction kit (LGC, Teddington, UK) and analyzed by Q5 PCR using the following primers: PGK-F1 or PGK-F2 and T1E2-HygroR6 and T1E2-HygroR7 (Supplementary Table 3). Correctly-targeted clones were checked for proper HOXA13 induction by the addition of 1.25 µg/ml doxycycline to the culture medium for 3 days. Three HOXA13 overexpression versus three control biological replicates were selected and used for experiments.

Differentiation of HK2 mouse embryonic stem cells. An optimized version of the Opgal protocol was used67. Cells were plated on 50% confluent pre-cultured M15 cells, a mesenchymal-derived feeder cell line67 (gift of N. Haste, University of Edinburgh, UK). Cells grew six days in differentiation medium consisting of ESC medium without LIF, with the addition of Activin-A, basic Fibroblast Growth Factor, CHIR, and Noggin (Supplementary Table 3). HOXA13 was induced on day four by doxycycline at 1.25 µg/ml. On day six, cells were analyzed by FACs by double staining with 0.8 µg PE Rat Anti-Mouse CD184 (CXCR4) and 2.0 µg Anti-CD324 Alexa Fluor® 488 (E-Cadherin) at 4°C for 45 min (Supplementary Table 3). The cells were analysed with a BD FACSCountTM II (BD Biosciences, USA). Data were analyzed with BD FACSDiva v8.0.1 software, which was obtained from BD Biosciences, and processed using Microsoft Excel. Example of analysis is provided in the Supplementary Fig. 11. Double-positive cells were sorted and cultured for another day with doxycycline at 1.25 µg/ml before harvesting and RNA isolation took place, using the picoPure RNA isolation kit (Thermo-Fisher Scientific).
alleles were affected by unique out-of-frame deletions were selected along with three control cell lines.

RNA-sequencing. The EPC2-hTERT samples (n = 8) were treated with the TrueSeq Stranded mRNA Library Prep Kit. Sequencing took place according to the Illumina TruSeq v3 protocol on an Illumina HiSeq2500 sequencer. Sample preparation and sequencing was performed at the Erasmus MC. Reads of 50 base-pairs were generated and mapped against reference genome hg19 with TopHat (version 2.0.10). Expression was quantified using HTSeq-count (0.6.1). Stranded libraries of the BAR-T (n = 6), and both non-differentiated and differentiated HOXA13 samples (n = 6 each) were prepared with the NEBNext Ultra RNA sample prep kit. Sequencing took place according to the Illumina NextSeq 500 protocol on an Illumina HiSeq2500 sequencer. Sample preparation and sequencing was performed at GenomeScan in Leiden, The Netherlands. Reads of 75 base-pairs were generated, mapped against reference genome hg19 or mm9 with Tophat (version 2.1.0), and quantified using HTSeq (version 0.6.1p1). Data were processed using R, version 3.2.573, in combination with the module DeSeq274. Generated FCs and p-values adjusted for multiple testing, i.e. q-values, were analyzed using Ingenuity Pathway Analysis (IPA) version 42012434, obtained from QIAGEN Inc. (Hilden, Germany)275.

We limited the number of genes analyzed to a maximum of 1000 by eliminating genes with a (relatively) low fold change if differentially expressed genes number was above 1000. The dataset cut-offs used were always a q value of 0.05, the fold change cut-off was set at: nondifferentiated KH2-mESCs, FC 2, 888 genes; differentiation of KH2-mESCs, FC 5, 924 genes; differentiated KH2-mESCs, FC not restricted. 146 genes; EPC2-hTERT, FC 1.3, 990 genes. Activity scores are known in IPA as “z-scores” which represents the number of standard deviations from the mean of a normal distribution. For analysis and visualization of gene expression in the epidermal differentiation complex the raw counts from both models normalized to total reads were used. Genes for which both cell models had less than ten reads in the context of the different samples were excluded. Overlap in multiple testing corrected differentially expressed genes in the BAR-T and EPC2-hTERT datasets was calculated as follows; the proportion of overexpressed genes in the EPC2-hTERT dataset was determined. Half of the differentially expressed genes in the BAR-T dataset would be expected to be regulated in the same direction. Regulation would be random. This expected overlap if regulation was random, and the observed overlap, were used as input for an \(X^2\) test. Information included in Supplementary Data 2 and 3 in the “known function” and “Detailed description” columns was obtained through non-systematic review and should not be considered as an exhaustive overview of the literature. Expression of molecules in the distal GI-tract with their known function and a two-sided Student's t-test. Information included in Supplementary Data 2 and 3 in the “known function” and “Detailed description” columns was obtained through non-systematic review and should not be considered as an exhaustive overview of the literature.

Acid and bile exposure. For assessment of HOXA13 mRNA expression upon acid/bile exposure, EPC2-hTERT and HET-1A cells were treated with 30 min cell culture medium adjusted to a pH of 7.0 or 4.0 using HCl. Cells were subsequently washed using PBS and given standard medium. A second time exposure was performed four times in duplo. Cells were separately exposed to medium with a bile acid mixture in concentrations of 0, 200, (and 400 for EPC2-hTERT) μM/L for 30 min at a pH of 7.0. The bile acid mixture consisted of 25% deoxycholic acid, 45% glycocholic acid and 30% taurocholate. Acid and bile exposure was performed twice in duplicate. After 24 h, the cells were harvested and RNA was isolated. Methods were derived from Bus et al.276. To assess the effect of bile/acid on expansion of cells, EPC2-hTERT cells transduced with HOXA13 or control vector as described above were seeded in 96-well plate with at least 2 wells per condition. Next day, medium was replaced with 100 μl of bile/acid mixture in cell culture medium (50 μM of sodium glycocholate, 50 μM taurocholate. Acid, pH=4.95). After incubation for 4 days, MTT test was performed as described below. Experiment was performed at least five times.

BAR-T spatial distribution experiments. These were performed with three biological replicates cell lines containing HOXA13 knock-out and three control cell lines. 400 80-BAR-T cells were seeded in a 6 well plate and pictures were taken the second day after seeding. Per well three pictures were taken. These pictures were analyzed using FIJI, using the multipoint tool, an X and Y (pixel) coordinate table was generated277. The distance between each cell and its three closest neighbors was quantified using Microsoft Excel and analyzed by two sided student’s t-test. The experiment was performed in three independent cell lines and repeated three times.

MTT assay. For assessment of cell growth of EPC2 and BAR-T cells, we performed a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay277. We seeded 1000 cells per well in 96 well plates for each of the three wild-type and three HOXA13 knock-out cell lines. Per condition at least 2 wells were used. On days one, three, five, and seven 10 μL MTT at 5 μg/ml was added and incubated for three hours, the medium was removed, and the precipitate was dissolved in 100 μL DMSO, which was incubated for five minutes under shaking. For BAR-T cells, absorption was measured in a BioRad microplate reader Model 680 XR at 490 and 595 nm, the average absorption was used to process the data. For EPC2 cells it was measured with Tecan microplate reader Model Infinite 200 pro at 565 nm with reference wavelength 670 nm. The experiment was repeated three times and a two sided Student’s t-test was used to test for statistical significance.

3D culture EPC2-hTERT cells. 3D culturing of EPC2-hTERT cells was performed as previously described28, 4000 EPC2-hTERT cells in culture medium were mixed 1:1 with ice-cold Matrigel basement membrane matrix (Corning BV), seeded in 50 μl drops in a 24 well plate for cell suspension, and incubated at 37°C for 30 min. After solidification, 500 μl of culturing medium supplemented with 0.6 mM CaCl2 was added. Y27632 (10 μM) was included in medium only the first 24 h after seeding. Medium was refreshed and pictures were taken every three days. The morphology of spheroids (based on number of extrusions, or ‘invadosomes’) was counted on day 5. The area of the spheroids was measured with Fiji28 for H&E staining and IHC. Three spheroids were excised with 5×104 cells embedded in 3D culture medium, 8H, 16H, 24H (see above). Sections were stained with 45 μM paraffin-embedded sections were deparaffinized, and stained with hematoxylin and eosin, PAS staining and immunohistochemistry for involucrin were performed.

Rat trachea in vivo tissue reconstitution model. 50,000 parental BAR-T cells (derived from six independent clones) or HOXA13 knock-out clones (three independent clones) in 30 μl of medium were seeded in the lumen of devitalized and denuded rat tracheas and implanted under the dorsal skin of NOD SCID gamma mice as described by Croagh et al.278. Mice were housed in microisolator cages with a 14 h light/10 h dark cycle, standard chow and water ad libitum, and temperature and humidity maintained at 21 ± 1°C and 50 ± 10%, respectively. Mice were sacrificed after four or six weeks. Harvested tracheas were formalin fixed, decalcified, embedded in paraffin and sectioned. Staining with hematoxylin and eosin, alcin blue, PAS staining and immunohistochemistry using antibodies against human mitochondria. CK7, TFF3, CDX2, p63, CK5, and involucrin (a gift from Prof. Pritinder Kaur, Curtin University, Australia or #59181-100UL from Sigma-Aldrich) were performed (Supplementary Fig. 9 and Table 4). These murine experiments were approved by the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee and were performed according to the guidelines of the same institution.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The RNA-seq data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus289 and are accessible through GEO Series accession number GSE17317081. There are no restrictions regarding data availability. Supplemental figures, tables, and a method are included. Source data are provided with this paper and all relevant data are available from the authors.

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Conceptualization, V.T.J., M.P.P., A.W., J.C., E.I.K., L.J.W.L., W.A.P., M.C.W.S., K.N., G.F. and R.S.; Methodology, V.T.J., G.M.F., M.P.P., R.A.S., N.C., R.S., K.N.; Formal Analysis, V.T.J., K.N., R.A.S.; Investigation, V.T.J., R.A.S., N.C., K.N.; Resources, M.C.W.S., H.S.S., E.M.T., T.S.G.; Writing – Original Draft, V.T.J., K.N. GMF and A.P.V.; Writing – Review & Editing, V.T.J., K.N., M.P.P., M.C.W.S., A.P.V., A.W., G.M.F., R.A.S., R.S., M.J.B., N.C., W.A.P., J.C., E.I.K., L.J.W.L., H.S.S., E.M.T., T.S.G., J.C., M.M., E.I.K., L.J.W.L., K.N.; Funding Acquisition, M.C.W.S., M.P.P., M.J.B., V.T.J., W.A.P.; Visualization, V.T.J., N.C., A.P.V., K.N.; Supervision, M.P.P., M.J.B., M.C.W.S., GMF.

Competing interests

M.J.B.: Cook Medical; consultant, support for industry and investigator initiated studies. Boston Scientific; consultant, support for industry and investigator initiated studies. Pentax Medical; support for investigator initiated studies. Mylan; support for investigator initiated studies. ChirOStim; support for investigator initiated studies. 3 M; support for investigator initiated studies. The remaining authors declare no competing interests.

Additional information

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