Deciphering Precursor Cell Dynamics in Esophageal Preneoplasia via Genetic Barcoding and Single-Cell Transcriptomics

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Abstract

Cancer cells exhibit high heterogeneity and lineage plasticity, complicating studies of tumorigenesis and development of therapies. Recently, preneoplastic cells, although histologically normal, have been shown to possess high plasticity and early genetic alterations, yet their origins and lineage trajectories remain unclear. Herein, we introduce a lineage-tracing tool integrating genetic barcoding with single-cell RNA sequencing to map preneoplastic esophageal cell lineages. We identified preneoplastic precursor cells (PNPCs) as a distinct progenitor-like population with unique transcriptional profiles and high plasticity, contributing to proliferative and basal cell populations. To enhance lineage mapping, we developed the eXamined Ridge (XR) score, accurately identifying high-plasticity cells. *Nfib* and *Qk* emerged as conserved PNPC markers, peaking in early preneoplasia and declining after malignant transformation. These findings reveal PNPCs as key players in early tumorigenesis and highlight their potential as biomarkers for early cancer detection and therapeutic intervention, offering new strategies for preventing esophageal cancer progression.

Introduction

Cancer cells exhibit extensive heterogeneity arising from diverse cell lineages, which contributes to drug resistance and tumor relapse.[1, 2] Understanding these cell lineages has long been a major challenge in understanding the biology of cancer and establishing effective therapeutic strategies. However, despite extensive studies of cancer cells, the identification of cancer stem cells and the related cell lineages remains elusive.[3, 4] This challenge is exacerbated by the clonal diversity within neoplastic cells, which intensifies cellular heterogeneity and complicates lineage relationships. Since intra-tumor heterogeneity arises from both genetic and non-genetic mechanisms from the early phase of tumor development, therapeutic strategies targeting genes or cells in late-stage tumors often fail to achieve durable tumor control.[5] Therefore, targeting core cell lineages during the early stages of tumor development, before clonal expansion, is crucial. Recent studies have also reported that preneoplastic cells acquire cell plasticity, granting them the potential to transition into neoplastic cells.[6-8] Elucidating the unique high-plasticity cell populations and lineage trajectories of preneoplastic cells, which are distinct from normal cells, could provide critical insights for preventing cancer development.

Experimental approaches used to identify cells of origin have included genetic reporter and barcoding systems.[9-11] Genetic reporters, such as those encoding fluorescent proteins or beta-galactosidase, have been widely used for permanent labeling of parent cells through spatiotemporal genetic recombination using Cre-loxP or Flp-Frt systems. When controlled by tissue- or cell-specific promoters, these genetic recombination systems enable precise tracking of cellular lineage.[12] Despite their broad applications for in vivo models, genetic reporter systems typically rely on a limited set of researcher-selected genes for cell labeling, which introduces potential bias.[13, 14] Moreover, the ability to trace multiple cell lineages is constrained by the limited number of available fluorescent tags with non-overlapping emission spectra.[11] Although stochastic and combinatorial expression models for fluorescent proteins have recently been introduced, the number of distinguishable colors remains restricted, and the persistence of fluorescence can present additional challenges.[15, 16] Another significant limitation is the ubiquitous expression of reporters across all cells within the same lineage. Thus, the genetic reporter-based approach captures only a single snapshot of lineage dynamics at a specific time point, making the interpretation of lineage directionality more subjective and potentially less reliable.

Genetic barcoding has also been used to track large numbers of cells by integrating diverse barcode libraries into DNA, which are subsequently inherited by daughter cells originating from stem cells.[17, 18] With advancements in next-generation sequencing, this technique has been successfully used to map cellular differentiation trajectories from stem cells.[19] Meanwhile, single-cell RNA sequencing (scRNA-seq) has addressed several limitations of earlier methods by enabling transcriptomic analysis at the single-cell level. Computational tools such as RNA velocity, CytoTRACE, and signaling entropy have been developed to reconstruct cell lineages based on transcriptional dynamics.[20-22] Although these tools have helped to reduce biases, they still have inherent limitations, as they rely on a restricted set of prior knowledge and predict cell lineages through computational inference, which may introduce uncertainties.

These significant advances notwithstanding, identifying stem cells and tracing the origins of cancer cells remain a formidable challenge. This difficulty primarily arises from the high cellular heterogeneity and plasticity of cancer cells, which can undergo de-differentiation or trans-differentiation—mechanisms that are rarely observed in normal cellular hierarchies.[1, 2, 23] These dynamic transitions obscure lineage relationships and complicate the identification of tumor-initiating populations. Therefore, novel tools are needed to accurately resolve these atypical cell states without overreliance on inference-based predictions or prior biological knowledge.

Esophageal tissue consists of relatively simple cell types, and its normal epithelial cell lineage has been well characterized. However, esophageal squamous cell carcinoma (ESCC) exhibits significant heterogeneity, with disrupted and ambiguous cell identities serving as a hallmark of this cancer type.[24] Studies of genomic data from patients with ESCC and experimental models have revealed that key genomic alterations occur before the onset of neoplastic tumor growth.[25-27] Although these genetic alterations alone are insufficient to drive malignant transformation, cells harboring them display transcriptomic profiles closely resembling those of neoplastic cancer cells, underscoring their critical role as a potential source of cancer development.[27]

In this study, we introduce a novel tool to overcome the current limitations of lineage tracing and identify the cells of origin in preneoplastic lesions, characterizing preneoplastic cells at an early stage represents a promising strategy for intercepting tumor progression and preventing the transition into fully malignant states. The findings from this study, which focus on identifying the origins of esophageal preneoplastic cells, provide valuable insights into potential strategies for improving the prognosis of patients with esophageal cancer by uncovering the early molecular events of neoplasia.

Results

Establishment of cell lineage networks by using iTracer vectors and scRNA-seq analysis

To identify the unique features of preneoplastic cells, we used a genetically engineered mouse esophageal organoid model for preneoplasia, as described previously.[27] The combinatorial knockout (KO) of *Trp53* and *Cdkn2a* results in organoids that maintain normal morphology while exhibiting transcriptomic profiles closely resembling those of neoplastic models, indicating a preneoplastic phenotype.[27] Briefly, esophageal organoids derived from *Trp53* $^{f/f}$ mice were infected with an adenoviral vector expressing Cre, followed by transduction with a CRISPR/Cas9 system targeting *Cdkn2a* using single guide RNA, thereby generating *Trp53 Cdkn2a* double KO (PC) organoids (Fig. 1A).[27-29] These PC organoids were subsequently cultured in a 2D system to assess their autonomous proliferative capacity in the absence of growth factor-enriched medium.

To trace the lineage of preneoplastic cells, we used iTracer, a lineage recorder that combines a genetic barcode with CRISPR/Cas9-induced scar systems.[30] This system integrates 11-nucleotide random DNA sequences into each cell, which are inherited by daughter cells. Over successive generations, CRISPR/Cas9-mediated genomic insertions or deletions (indels) accumulate, allowing the reconstruction of cellular hierarchies and lineage relationships.

The iTracer plasmid library was prepared as previously described and transfected into 2D-cultured preneoplastic PC cells (Fig. 1B).[30] iTracer-introduced PC cells were isolated via flow cytometry and subjected to single-cell RNA sequencing (scRNA-seq). The reference genome was specifically modified to include sequence information for barcode and scar regions, enabling precise lineage tracing through unique genetic markers (Fig. 1C). From the transcriptomic profiles, we identified 5,167 out of 17,703 cells exhibiting detectable green fluorescence protein (GFP) transcripts, confirming successful expression of the transduced barcodes and scars (Fig. 1C). Barcodes and scars were extracted from the GFP-positive cells by using Samtools, facilitating the construction of lineage networks and enabling a detailed analysis of cellular trajectories and clonal relationships (Fig. 1C).

Single-cell transcriptomics-based dissection of cell clusters in esophageal preneoplasia

We used scRNA-seq and identified seven distinct cell-type clusters based on marker gene expression, with one cluster remaining undefined (Fig. 2A-C and Supplementary Fig. 1A). The proliferating cell cluster exhibited significant G2M enrichment, indicating active cell division (Fig. 2D), whereas the undefined cluster showed an unexpected accumulation of unspliced transcripts, suggesting an impairment in mRNA splicing (Fig. 2D). Gene set enrichment analysis and gene scoring analyses revealed a consistent downregulation of mRNA splicing pathways in the undefined cluster (Fig. 2E and Supplementary Fig. 1B-C). We also observed upregulation of the G2M checkpoint in the proliferating cluster and increased expression of the epithelial-to-mesenchymal transition (EMT) pathway in the EMT-like cluster (Fig. 2F). Notably, the undefined cluster exhibited downregulation of apoptosis pathways, ruling out the possibility that it represents a population of dead cells (Fig. 2F). Further regulatory network analysis revealed a high activation of multiple regulons in the undefined cluster (Fig. 2G), with the top four being *Mtf1, Zbtb24, Tlx3,* and *Thap1*. Among these, *Mtf1* has been reported to promote tumor progression by activating downstream targets,[31, 32] whereas *Tlx3* has been shown to be associated with cell self-renewal and cancer stemness, particularly in prostate cancer.[33, 34] These findings suggest that the undefined cell cluster may have a crucial role in tumorigenesis.

Construction of cellular lineage network based on barcode and scar information

To investigate cellular lineage, we used CytoTRACE and RNA velocity, scRNA-seq-based cell lineage trajectory inference tools.[21, 35, 36] By using these inference-based cell trajectory analyses, we found that the cellular lineage originated from the proliferating cell cluster and transitioned toward other cell types, including basal, suprabasal, and differentiated cells (Fig. 3A-C). Notably, a strong lineage trajectory toward the EMT-like cell cluster was observed (Fig. 3A), a feature absent in normal esophageal epithelium. Although these analyses revealed some clear cell trajectories, the undefined cluster exhibited an isolated and distinct pattern compared with the other clusters, suggesting a unique cellular trajectory that was not evident in the inference-based analyses (Fig. 3A-C and Supplementary Fig. 2A). A PHATE (potential of heat-diffusion for affinity-based trajectory embedding) map[37] further supported the distinct characteristics of the undefined cluster, as it remained separated from other clusters (Fig. 3D).

To identify the connections between the undefined cluster and other cells, we analyzed barcode and scar data and found 497 cellular lineage links between cells sharing the same barcode (Fig. 3E). Strikingly, the undefined cell cluster exhibited the lowest number of scar types, yet a substantial portion of lineage links from the undefined cluster was directed toward other cell types, with the strongest signals observed in the proliferating and basal cell populations (Fig. 3E, F and Supplementary Fig. 2B, C). This observation suggests that the undefined cells represent preneoplastic precursor cells (PNPCs) that give rise to other cell types, including proliferating and basal cells.

To further delineate the cellular lineage in preneoplastic cells, we constructed a cellular lineage network with barcode and scar-based links and calculated a network score based on the number of connections between clusters. This analysis revealed a strong trajectory from PNPCs to basal and proliferating cell clusters, with relatively weaker connections to other cell types (Fig. 3G, H). These results indicate that PNPCs are more closely related to proliferating and basal cells than to other cell populations.

Although the network score provided direct evidence of the PNPC lineage leading to proliferating and basal cells, the overall map of cell lineages and their sequential orders remained unclear. To address this, we applied the network score to the traditional valley-ridge (VR) score method[38, 39] and developed the eXamined Ridge (XR) score, as outlined in the Methods (Supplementary Fig. 2D). The XR score provided a more precise representation of the undefined cluster as progenitor cells with the highest cellular plasticity, which was visualized on Waddington's landscape (Fig. 3I and Supplementary Fig. 2E).

In contrast, the VR score, which relies on inference-based approaches, failed to capture the high plasticity of PNPCs. Moreover, our XR score more effectively distinguished other parts of the preneoplastic cell lineage, such as the basal/proliferating-suprabasal-EMT-like cell axis, than did the VR score in both PHATE (Supplementary Fig. 2E, F) and uniform manifold approximation and projection (UMAP) embeddings (Supplementary Fig. 2G, H).

Identification of PNPCs-specific markers

To identify molecular markers for detecting the progenitor cell cluster, we used differential gene expression analysis to compare the progenitor cell cluster with other clusters. Interestingly, the expression of marker genes in the progenitor cell cluster exhibited relatively low "fold-change" values compared with marker genes from other clusters, highlighting its quiescent, progenitor-like characteristics (Fig. 4A).

To refine the identification of specific markers, we conducted additional scRNA-seq analysis on normal human esophageal epithelial cells and normal adjacent cancer (NAC) cells from patients with esophageal squamous cell carcinoma (ESCC).[40-43] NAC cells exhibit intermediate characteristics between normal and tumor cells and were therefore considered to include preneoplastic cells. As expected, NAC cells displayed several distinct cell clusters on a UMAP after integration with normal esophageal epithelial cells, suggesting that their cell identities had shifted from a normal state (Fig. 4B).

From the intersection of these analyses, we identified a set of potential markers. Among these, we specifically focused on genes with known oncogenic functions, as defined by the Cancer Gene Census,[44] a comprehensive database cataloging genes with strong experimental and clinical evidence of involvement in cancer. The Cancer Gene Census classifies genes based on their roles as oncogenes or tumor suppressors, considering factors such as somatic mutations, germline predisposition, and functional studies. To refine our selection, we prioritized genes with established oncogenic activity, particularly those recurrently mutated, amplified, or overexpressed in cancer, leading to the identification of 26 candidate markers of PNPCs (Fig. 4C and Supplementary Fig. 3). Among these, *Nfib* and *Qk* were selected as consistent markers in both mouse and human models not only because of their significantly elevated expression in PNPCs and NAC cells but also because high-specificity antibodies were available for their detection. This enabled precise experimental validation, which allowed accurate localization and functional characterization in preneoplastic cells (Fig. 4C-F and Supplementary Fig. 4A, B).

We further validated the increased expression profiles of *Nfib* and *Qk* by analyzing scRNA-seq data from various experimental models. First, we examined scRNA-seq data from 3D organoids of wild-type (WT) cells, *Trp53* and *Cdkn2a* double-KO (PC) cells, and *Trp53, Cdkn2a*, and *Notch1* triple-KO (PCN) cells. *Nfib* and *Qk* expression was upregulated in PC organoids but slightly decreased in PCN organoids (Fig. 4G-I and Supplementary Fig. 4C). These findings were further validated with immunohistochemical staining of normal esophageal tissue, PC-derived preneoplastic (benign) tumors, and PCN-derived neoplastic (malignant) tumors. Both QKI and NFIB expression were elevated in benign tumors but slightly reduced in neoplastic tumors (Fig. 4J). Because PC and PCN organoids represent preneoplasia and early neoplasia, respectively,[27] these results suggest that *Nfib* and *Qk* are specifically upregulated during the preneoplastic stage of cancer progression.

We also analyzed scRNA-seq data from a carcinogen-induced mouse ESCC model. The expression of *Nfib* and *Qk* was upregulated during the early stages of cancer progression, especially in the inflammation stage. However, their expression gradually declined as the cells progressed toward neoplastic transformation and increased again at the cancer in situ stage (Fig. 4K-M and Supplementary Fig. 4D).

This dynamic pattern was also consistent in human samples across different disease stages. A significant upregulation of NFIB and QKI expression was observed during the inflammation, hyperplasia, and cancer in situ stages, whereas a decline was noted in the later stages of ESCC (Fig. 4N, O and Supplementary Fig. 4E, F).

These results demonstrate that PNPCs expand during the early stages of tumorigenesis, even though they are histologically classified as normal cells. However, these precursor cells decrease in number after [the?] transformation into neoplastic cells. These findings also imply that PNPCs might be essential for overcoming selection pressures during the initial stages of tumor development but become less critical for clonal expansion (Fig. 4P).

Discussion

This study advances our understanding of esophageal preneoplasia by identifying preneoplastic progenitor cells (PNPCs) and their cellular dynamics. By combining genetic barcoding with scRNA-seq, we established a comprehensive cellular lineage map of esophageal preneoplastic cells. This approach allowed us to overcome key limitations of scRNA-seq-based lineage analysis, which primarily relies on inference-based predictions or prior biological knowledge. Although inference-based analyses have been effective in delineating cell lineage trajectories in normal tissues, they often overlook unknown cell types that are critical to transformed cell populations owing to the lack of direct experimental validation. Here we identified lineage connections through experimental validation, revealing a specific cell cluster functioning as a progenitor population that gives rise to proliferating and basal cells. This finding was further reinforced by the development of the eXamined Ridge (XR) score, which highlighted the distinct role of this progenitor cluster in driving cellular heterogeneity during early neoplastic transformation.

Preneoplastic cells have gained increasing attention because of the transcriptional and epigenetic reprogramming that occurs at this stage, even though these cells remain phenotypically normal.[45, 46] During this early phase of tumorigenesis, cells acquire plasticity, leading to the emergence of distinct progenitor cell populations.[8] Most of the recent studies on precancerous stages of esophageal squamous cell carcinoma (ESCC) have focused on dysplastic stages—later points in disease progression—making them less suitable for understanding the initial events of tumorigenesis.[47-49] In contrast, our study focused on earlier cellular events, where only two genetic alterations (*Trp53* and *Cdkn2a* loss) were introduced. Although these genetic alterations are commonly observed in patients with ESCC, research on them has been limited, as they alone are insufficient to drive neoplastic transformation. However, as shown in Figure 3, *Trp53* and *Cdkn2a* loss (the PC model) was sufficient to generate PNPCs, which exhibited the highest cellular plasticity.

Intriguingly, PNPCs displayed unique features, including suppressed mRNA splicing and high activation of Mtf1, Zbtb24, Tlx3, and Thap1 regulons. Because these regulons have critical roles in tumor progression, self-renewal, and DNA methylation, it is highly plausible that PNPC clusters serve as a key source of cellular plasticity in early ESCC tumorigenesis. We also identified two transcriptional markers, *Nfib* and *Qk*, that were enriched in PNPC clusters and had critical roles in cancer progression. These markers were validated across several experimental models, including 3D organoids, mouse tissue models, and human ESCC samples. Interestingly, their expression patterns were dynamic, showing upregulation during early preneoplasia, downregulation during cellular expansion, and re-emergence during cancer in situ (Fig. 4L). Eventually, their expression slightly decreased again in later ESCC stages (Fig. 4O). Although the dynamics of PNPCs are not entirely consistent between mouse and human temporal samples, a clear trend emerged: PNPCs are significantly expanded during the early stages of

both preneoplasia and neoplasia. This expansion suggests that PNPCs provide the cellular plasticity required to overcome early selection pressures, ensuring their survival and adaptation during the initial stages of tumorigenesis.

The dynamic expression pattern of *NFIB* and *QKI* suggests that they may have dual roles in early preneoplasia and neoplasia. Given the known functions of NFIB as a transcription factor and QKI as a mRNA splicing regulator, their roles are likely crucial for modulating gene expression that is required for cellular adaptation to the microenvironment during these early stages. Further studies are needed to elucidate their precise regulatory mechanisms across different stages of preneoplasia and cancer progression.

Beyond PNPCs, we also identified an EMT-like cell cluster, which was absent in the normal esophagus (Fig. 3). Interestingly, this EMT cluster originated from progenitor cells (proliferating or basal cells) or PNPCs but not from differentiated cells. This suggests that the emergence of mesenchymal-like differentiation in preneoplastic esophageal cells was previously unrecognized. Our findings indicate that preneoplastic esophageal cells diversify cell lineages, generating cellular heterogeneity beyond the normal epithelial trajectory.

Despite the homogeneous genetic alterations in our preneoplasia model (*Trp53* and *Cdkn2a* loss), we observed highly variable and heterogeneous cell fates (Supplementary Fig. 4G, H). This observation suggests that cancer evolution in preneoplastic cells is not solely governed by genetic changes but is also influenced by non-genetic mechanisms, such as epigenetic factors and tumor niche.[50, 51] Although our model effectively captured autonomous cellular and molecular events in preneoplastic cells, it did not account for selection pressures imposed by the microenvironment, such as interactions with the immune niche. In addition to the effect of the tumor microenvironmental niche, intra-tumoral selection processes need to be verified in animal models to demonstrate the role of PNPCs during tumorigenesis. Although our study provides a detailed snapshot of preneoplastic cells, their roles in cancer evolution can be more comprehensively understood through in vivo models. To address these limitations, our future studies will focus on exploring the roles of the microenvironment and intra-tumoral events in the transition to malignancy by using genetically engineered mouse models.

In summary, our study provides a comprehensive cellular and molecular framework for understanding esophageal preneoplasia by identifying a progenitor cell cluster with distinct transcriptional signatures. Our findings highlight the dynamic cell lineage relationships that drive early cancer progression, offering a foundation for future investigations into tumor initiation. By uncovering key molecular markers associated with preneoplastic transformation, this study also provides valuable insights for refining ESCC risk stratification and developing early diagnostic strategies.

Author contributions

J.J.: Conceptualization, methodology, investigation, software, analysis, data curation, writing (original draft), visualization; K.-P.K.: Conceptualization, methodology, investigation, software, analysis, data curation, writing (original draft), visualization, funding acquisition; J.Z.: Investigation; S.J.: Investigation; J.-I.P.: Conceptualization, methodology, analysis, writing (review and editing), visualization, supervision, project administration, funding acquisition

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Declaration of interests

The authors declare no competing interests.

Materials and methods

Cell culture

PC (*Trp53 Cdkn2a* double knockout [KO]) and PCN (*Trp53 Cdkn2a Notch1* triple KO) organoids and 2D cells were prepared as previously described.[27] Briefly, esophageal epithelial cells isolated from *Trp53*$^{floxed/floxed}$ mice were used to establish esophageal organoids. Ad-Cre-EGFP (University of Iowa) was introduced to develop Trp53$^{-/-}$ organoids. Next, CRISPR/Cas9 system-mediated *Cdkn2a* or *Notch1* KO was added to the Trp53$^{-/-}$ organoids to establish the PC and PCN organoids. Organoid cells were dissociated into single cells and cultured on 24-well plates with Dulbecco's Modified Eagle Medium (DMEM) + 10% fetal bovine serum (FBS) with 10 μM Y-27632. After the third passage, cells were cultured without Y-27632.

Barcode library preparation

Barcode libraries were prepared as described elsewhere.[30] iTracer plasmid (#351) was purchased from the European Plasmid Repository. Barcode sequences were amplified by using a custom-designed barcode template and primers: Barcode-template_perturb, 5'-ACGAGCTGTACAATAAGCTGATCCWNNNNNNNNNWCCAAGCGCCCTTGAGCATCTG-3'; Barcode forward, 5'-GGACGAGCTGTACAAGTAAGCTG-3'; Barcode reverse, 5'-GAATCAGATGCTCAAGGGCGCTTG-3'. Amplified barcodes were purified and ligated with NotI-linearized iTracer plasmid by using an NEBuilder HiFi DNA Assembly kit. The ligation product was transformed into DH5α cells, which were spread on LB plates containing ampicillin and incubated overnight at 37°C. Colonies were then scraped from the LB plates and pooled in a 2× YT medium for plasmid extraction by using the EndoFree Plasmid Maxi Kit (Qiagen).

Transfection and sequencing library preparation for scRNA-seq

PC cells with constitutive expression of Cas9 were transfected with a prepared barcode library by using Lipofectamine 3000. Cells were amplified over three passages and subjected to flow cytometry and scRNA-seq. For flow cytometry, 1 million RFP-positive and live cells were sorted and prepared for scRNA-seq. Targeted cell recovery was approximately 20,000 cells. After the cDNA library was prepared, the gene expression library was made by using a 10x Genomics 3' v2 kit (Baylor College of Medicine), and barcode and scar libraries were generated separately. The barcode region was amplified by 27 cycles of root PCR (Forward, 5'-GACGACGGCAACTACAAGACC-3'; Reverse, 5'-CTACACGACGCTCTTCCGATCT-3') and 10 cycles of nested PCR for Barcode (Forward, 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGATCACTCTCGGCATGGA-3'; Reverse, 5'-CTACACGACGCTCTTCCGATCT-3') and Scar (Forward, 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGCCAACTTCAAGATCCGCC-3'; Reverse, 5'-CTACACGACGCTCTTCCGATCT-3') regions. The PCR product was purified by using 1× AMPure XP beads (Beckman). Indexing PCR for barcode and scar was conducted for 14 cycles by using dual-index primers from the 10x Genomics. Indexed libraries were purified by using 0.6× SPRI [solid-phase reversible immobilization] bead purification and quantified. The three libraries were sequenced for gene expression (400M reads), scar regions (167M reads), and barcode sequences (100M reads) on an Illumina NovaSeq (Novogene), and the fastq files were mapped to the customized reference genome by using CellRanger (10x Genomics Cloud).

Preprocessing scRNA-seq data

scRNA-seq data were preprocessed by using Scanpy (version 1.10.4).[52] Quality control (QC) steps were applied to filter cells and genes with low expression levels. Specifically, cells expressing fewer than 100 genes were removed, and genes detected in fewer than 3 cells were filtered out. After QC, only cells with

extractable barcode information from the alignment BAM files were selected for downstream analysis. This ensured that the dataset included cells with both transcriptomic and lineage information, which is critical for accurate lineage reconstruction. For subsequent analyses, including the identification of new cell populations and their unique markers, all genes passing the QC criteria were used to ensure a comprehensive and unbiased discovery process.

Barcode and scar analysis with LINNAEUS

Barcode sequences were analyzed with the LINNAEUS system by amplifying the regions flanking the barcode, including upstream and downstream sequences, and aligning them to the reference genome (mm10) by using the web-based Cell Ranger platform.[53] Cells were assigned to the same lineage only if they possessed identical barcode sequences, ensuring high specificity in lineage reconstruction. For each pair of cells with the same barcode, scar information was extracted from the alignment BAM files by using Samtools (version 1.21),[54] which analyzed insertion and deletion patterns encoded in the CIGAR string. The accumulation of scars was compared between cell pairs to infer differentiation directionality, with the assumption that scars progressively accumulate in more differentiated cells. This approach provided detailed insights into lineage relationships and cellular trajectory within the population.

Cell lineage inference

To infer cellular lineage trajectories, several complementary approaches were used. Cell cycle phases were inferred by using a curated list of cell cycle markers as described elsewhere,[55] enabling the identification of cells in G1, S, or G2/M phases. Pseudo-time analysis was conducted by using CytoTRACE (version 0.3.3) to estimate differentiation potential along the inferred trajectory.[21] RNA velocity analysis was done with Velocyto[35] and scVelo[36], which provided dynamic insights into cell state transitions based on spliced and unspliced mRNA ratios. To further analyze the directionality and entropy of cellular transitions, SCENT[22] was used to calculate cellular entropy, which was integrated with RNA velocity data to compute a Valley-Ridge (VR) score.[38] This VR score quantified differentiation potential and lineage progression for each cell cluster. Finally, a Waddington-like landscape was visualized by using Houdini, where the VR score was plotted along the vertical axis to represent differentiation potential, and the pseudo-time coordinates were mapped onto PHATE[37] coordinates representing pseudo-temporal progression on the horizontal axes with the VR score on the vertical axis. This integrated approach provided a detailed view of lineage relationships and the differentiation dynamics within the cell population.

Identification of molecular markers of PNPCs

To identify molecular markers specific to PNPCs, we conducted integrative analyses of scRNA-seq datasets from mouse cell lines and human esophageal cancer patient samples. In the mouse dataset, genes upregulated in the PNPC cluster were compared with other clusters to generate a PPC-specific gene list. For human data, samples from patients with esophageal cancer were subjected to scRNA-seq analysis. Differentially expressed genes were identified by comparing NAC samples with normal tissue. The PPC-specific gene list from the mouse dataset was intersected with the NAC-derived differential gene expression list to identify conserved candidate markers across species. To prioritize markers with oncogenic relevance, the resulting list was cross-referenced with the oncogenic gene list defined by the Cancer Gene Census.[44] This analysis identified 26 candidate PNPC markers, representing conserved molecular signatures with potential roles in early esophageal cancer progression. These markers were subsequently used for further validation and functional studies.

Calculating of XR scores

XR scores were calculated by integrating the VR score and the Network Score (NS) to represent lineage dynamics and differentiation potential. The NS was derived from a lineage network constructed from

barcode and scar information. For each cluster, the NS was defined as the number of differentiation cell pairs directed toward other clusters within the network, quantifying the cluster's contribution to lineage transitions.

Both the VR score and NS were normalized to ensure comparability across clusters by using the following formulas:

$$N_{VR_x} = \frac{VR_x - min\ (VR)}{max(VR) - min\ (VR)}$$

$$N_{NS_x} = \frac{NS_x - min\ (NS)}{max(NS) - min\ (NS)}$$

The XR score was then calculated as:

$$XR\ Score = \sqrt{((1-a)\ \times (N_{VR_x}))^2 + (a\ \times (N_{NS_x}))^2}$$

Here, a is a weighting coefficient that balances the contributions of the VR score and NS. To determine the optimal a, coefficients were tested from 0.5 to 0.9 in increments of 0.1. For each coefficient, the standard deviation of XR scores across clusters was calculated. Then, the median of these standard deviations across all clusters was computed to produce a single value for each coefficient. The coefficient that resulted in a median standard deviation of at least 0.01 was selected, ensuring consistent and robust differentiation patterns while avoiding excessive homogeneity in XR scores (Supplementary Fig. 2D). The resulting XR scores were visualized by using Houdini to create a Waddington-like landscape, with the XR score plotted on the vertical axis and PHATE coordinates representing pseudo-temporal progression on the horizontal axes. This visualization enabled a detailed interpretation of cellular differentiation trajectories and lineage relationships.

Human and mouse scRNA-seq dataset analyses

Mouse esophageal tissue datasets were obtained from the authors of the previous study.[56] Human healthy normal esophageal tissues and NAC datasets were downloaded from the Human Cell Atlas Data Portal (PRJEB31843) and National Center for Biotechnology Information Sequence Read Archive public databases (PRJNA828562, PRJNA672851, and PRJNA777911).[40-43]

Allograft transplantation and tissue isolation

Five-week-old C57BL/6 mice were maintained in the Department of Veterinary Surgery and Medicine at MD Anderson. 2D-cultured PC or PCN cells (3×10^6) were injected subcutaneously into the right dorsal flanks of mice. Animals were euthanized, and PCN and PC tumors were collected 100 days and 140 days after transplantation, respectively. The excised tumors were fixed and paraffin-embedded for immunostaining. To isolate normal esophagus tissue, 8-week-old mice were euthanized and subjected to cervical dislocation. Longitudinally opened esophagi were washed with phosphate-buffered saline (PBS), fixed, and paraffin-embedded. All animal procedures followed the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care and institutional (MD Anderson) approved protocols. This study was compliant with all relevant ethical regulations regarding animal research.

Immunohistochemical analysis

For human esophagus analysis, tissue microarray slides from patients with disease at different stages

were purchased from TissueArray.com. Immunostaining was done as previously described.[27] Briefly, paraffin-embedded tissue antigens were retrieved with a basic antigen retrieval buffer. Tissues were then blocked with goat serum in PBS and incubated with primary antibodies to QKI and NFIB (both from Proteintech, 1:200). The immunohistochemical staining results were scored, analyzed, and visualized by using GraphPad Prism (v10).

Data availability

scRNA-seq data are available via the GEO database (GSE289049).

Code availability

The code used to reproduce the analyses described in this manuscript can be accessed via GitHub (https://github.com/jaeilparklab/Esophagus_PNPC) and will be available upon request.

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Figure Legends

Figure 1. Barcoding and lineage tracing of esophageal preneoplastic cells.
(**A**) Schematic of the experimental workflow for generating mouse esophageal preneoplastic cells. KO, knockout.
(**B**) Schematic representations of analytic pipelines for barcode- and scar-based lineage tracing.

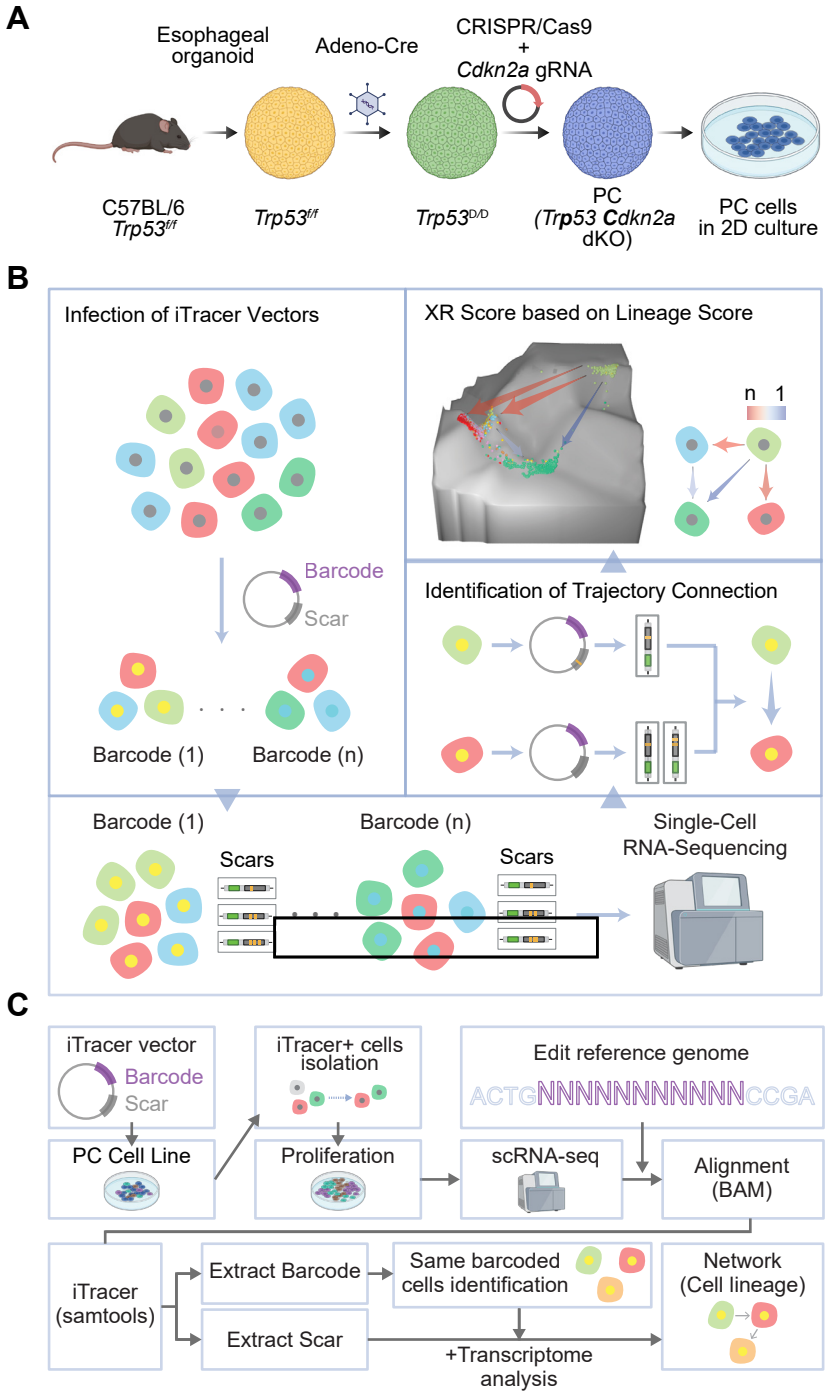
Figure 2. Characterization of cell clusters of preneoplastic cells.
(**A**) Uniform manifold approximation and projection (UMAP) with annotated cell clusters. EMT, epithelial-to-mesenchymal transition.
(**B**) Heatmap showing distinct transcriptional profiles across cell clusters.
(**C**) Heatmap displaying key marker gene expression across cell types, with scores from the rank_genes_groups function in Scanpy colored brown (high) and purple (low).
(**D**) Cell cycle phase analysis, indicating the distribution of cells in G1, G2/M, and S phases across different clusters.
(**E**) Gene set enrichment analysis demonstrating a significant downregulation of mRNA splicing pathways in the undefined cluster ($P = 0.02300$, NES = –1.3371) and minor mRNA splicing pathways ($P = 0.01406$, NES = –1.5493).
(**F**) UMAP (left) and violin plot (right) illustrating differential activation of key pathways across cell clusters.
(**G**) Heatmap displaying regulon analysis results, highlighting highly activated transcriptional regulators in the undefined cluster. Violin plots visualize the top four enriched factors: *Mtf1*, *Zbtb24*, *Tlx3*, and *Thap1* (Student's *t* test, ***$P<0.001$; 261 cells for undefined and 4,907 cells for others).

Figure 3. Identification of precursor cells through barcode-based cell tracing.
(**A**) UMAP with RNA velocity analysis results depicting inferred cellular trajectories based on transcriptional dynamics. Arrows indicate predicted lineage progression.
(**B**) UMAP displaying CytoTRACE analysis results, in which higher scores indicate lower differentiation states across cell populations.
(**C**) Violin plots showing CytoTRACE score distribution across cell clusters.
(**D**) PHATE visualization of cellular differentiation states.
(**E**) UMAP illustrating differentiation pathways based on barcode and scar-based lineage tracing, highlighting directional transitions between cells with shared barcode information across clusters.
(**F**) Box plot illustrating the distribution of CRISPR/Cas9-induced scars across cell populations. The box represents the interquartile range (IQR), with the median shown as a horizontal line. Error bars indicate [SD/SEM] to show data variability.
(**G**) Network-based lineage analysis illustrating cellular differentiation pathways based on barcode and scar information, with edge width reflecting the number of differentiation events.
(**H**) Schematic representation of differentiation flow, in which distances between cell types correspond to network scores.
(**I**) Waddington landscape illustrating cell lineage dynamics, comparing VR and XR scores to depict differentiation trajectories.

Figure 4. Expression of preneoplastic precursor cell markers in mouse and human esophagus.
(**A**) Box plot showing log(fold change) in significant markers ($P<0.05$) across cell clusters. The box represents the interquartile range (IQR), with the median shown as a horizontal line. Error bars indicate [SD/SEM] to show data variability.
(**B**) UMAP of healthy normal and normal adjacent cancer (NAC) human datasets. Proportions of normal and NAC cells in each cell cluster are displayed with bar plots.

(**C**) Venn diagram illustrating the overlap among preneoplastic precursor cell (PNPC) markers, NAC markers, and Cancer Gene Census (CGC) genes. A total of 26 genes are shared among all three groups.
(**D**) Violin plots showing the expression profiles of *Nfib* and *Qk* across different cell clusters.
(**E**) Dot plots of *NFIB* and *QKI* expression in human normal and NAC datasets.
(**F**) Violin plots illustrating expression levels of *NFIB* and *QKI* across healthy normal and NAC human datasets. (Student's *t* test, ***$P<0.001$).
(**G**) UMAP of genetically engineered mouse esophageal organoid datasets. WT, wild-type; PC, *Trp53 Cdkn2a* double knockout (KO); PCN, *Trp53 Cdkn2a Notch1* triple KO.
(**H-I**) Feature plots (**H**) and dot plots (**I**) of *Nfib* and Qk expression.
(**J**) Immunostaining of *QKI* and *NFIB* expression in mouse esophagus tissue and allograft tumors from PC and PCN cells.
(**K**) UMAP of carcinogen (4-NQO)-induce mouse esophageal tissue datasets.
(**L**) Feature plots of *Nfib* and *Qk* expression in mouse tissue datasets.
(**M**) Linear regression plot showing the expression levels of *Nfib* and *Qk* along the differentiation trajectory in mouse tissue datasets.
(**N-O**) Immunostaining (**N**) and quantification (**O**) of *QKI* expression in different stages of human esophageal disease (Student's *t* test, *$P<0.05$).
(**P**) Schematic representation of preneoplastic precursor cell expansion during cancer progression.

Figure 1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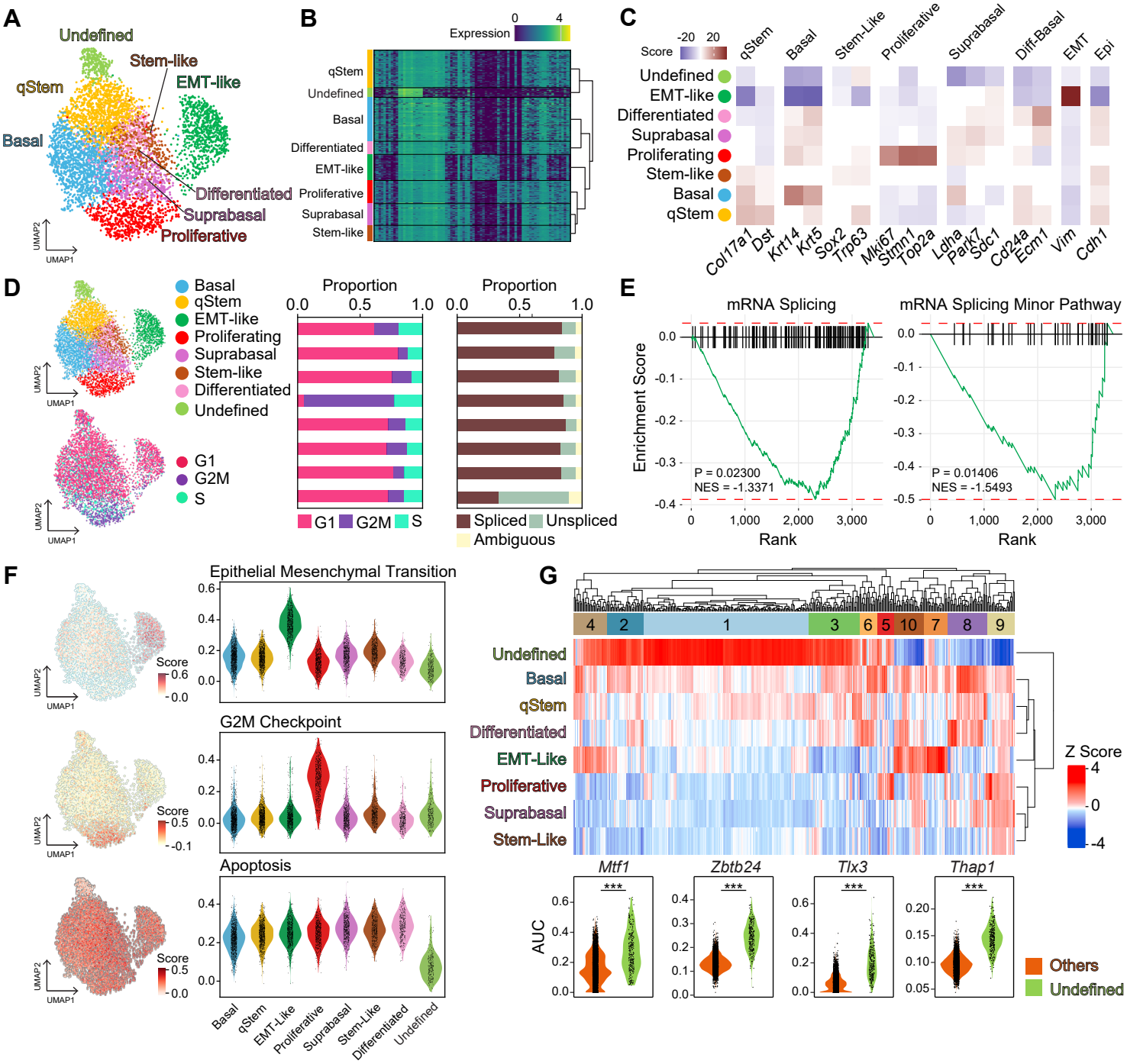
CRISPR/Cas9 + Cdkn2a gRNA
C57BL/6 Trp53f/f
Trp53f/f
Trp53D/D
PC (Trp53 Cdkn2a dKO)
PC cells in 2D culture
B
Infection of iTracer Vectors
Barcode
Scar
Barcode (1)
Barcode (n)
XR Score based on Lineage Score
n
1
Identification of Trajectory Connection
Barcode (1)
Barcode (n)
Scars
Scars
Single-Cell RNA-Sequencing
C
iTracer vector
Barcode
Scar
iTracer+ cells isolation
Edit reference genome
ACTGNNNNNNNNNNNNCCGA
PC Cell Line
Proliferation
scRNA-seq
Alignment (BAM)
iTracer (samtools)
Extract Barcode
Extract Scar
Same barcoded cells identification
+Transcriptome analysis
Network (Cell lineage)

Figure 2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EMT-like
Proliferative
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Proliferating
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Basal
qStem
Col17a1
Dst
Krt14
Krt5
Sox2
Trp63
Mki67
Stmn1
Top2a
Ldha
Park7
Sdc1
Cd24a
Ecm1
Vim
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Stem-like
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Undefined
G1
G2M
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Proportion
0.0
0.5
1.0
G1
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S
Spliced
Unspliced
Ambiguous
E
mRNA Splicing
mRNA Splicing Minor Pathway
Enrichment Score
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NES = -1.3371
P = 0.01406
NES = -1.5493
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2,000
3,000
F
Epithelial Mesenchymal Transition
G2M Checkpoint
Apoptosis
Score
Basal
qStem
EMT-Like
Proliferative
Suprabasal
Stem-Like
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Zbtb24
Tlx3
Thap1
AUC
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Others
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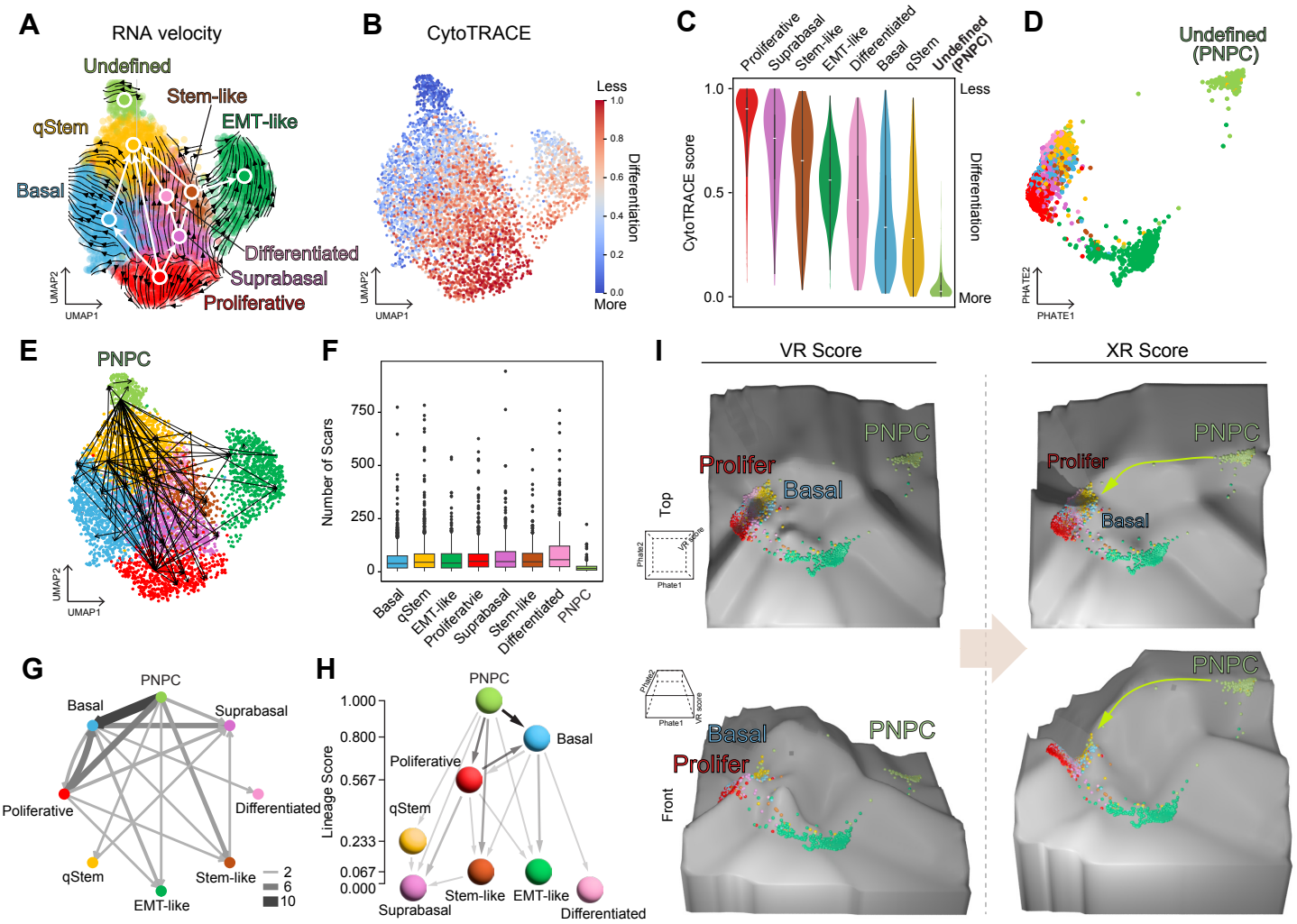
Figure 3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Proliferative
UMAP2
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CytoTRACE
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Differentiation
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C
Proliferative
Suprabasal
Stem-like
EMT-like
Differentiated
Basal
qStem
Undefined (PNPC)
CytoTRACE score
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Differentiation
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PHATE2
PHATE1
E
PNPC
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Number of Scars
Basal
qStem
EMT-like
Proliferatvie
Suprabasal
Stem-like
Differentiated
PNPC
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PNPC
Basal
Suprabasal
Poliferative
Differentiated
qStem
Stem-like
EMT-like
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PNPC
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Poliferative
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Lineage Score
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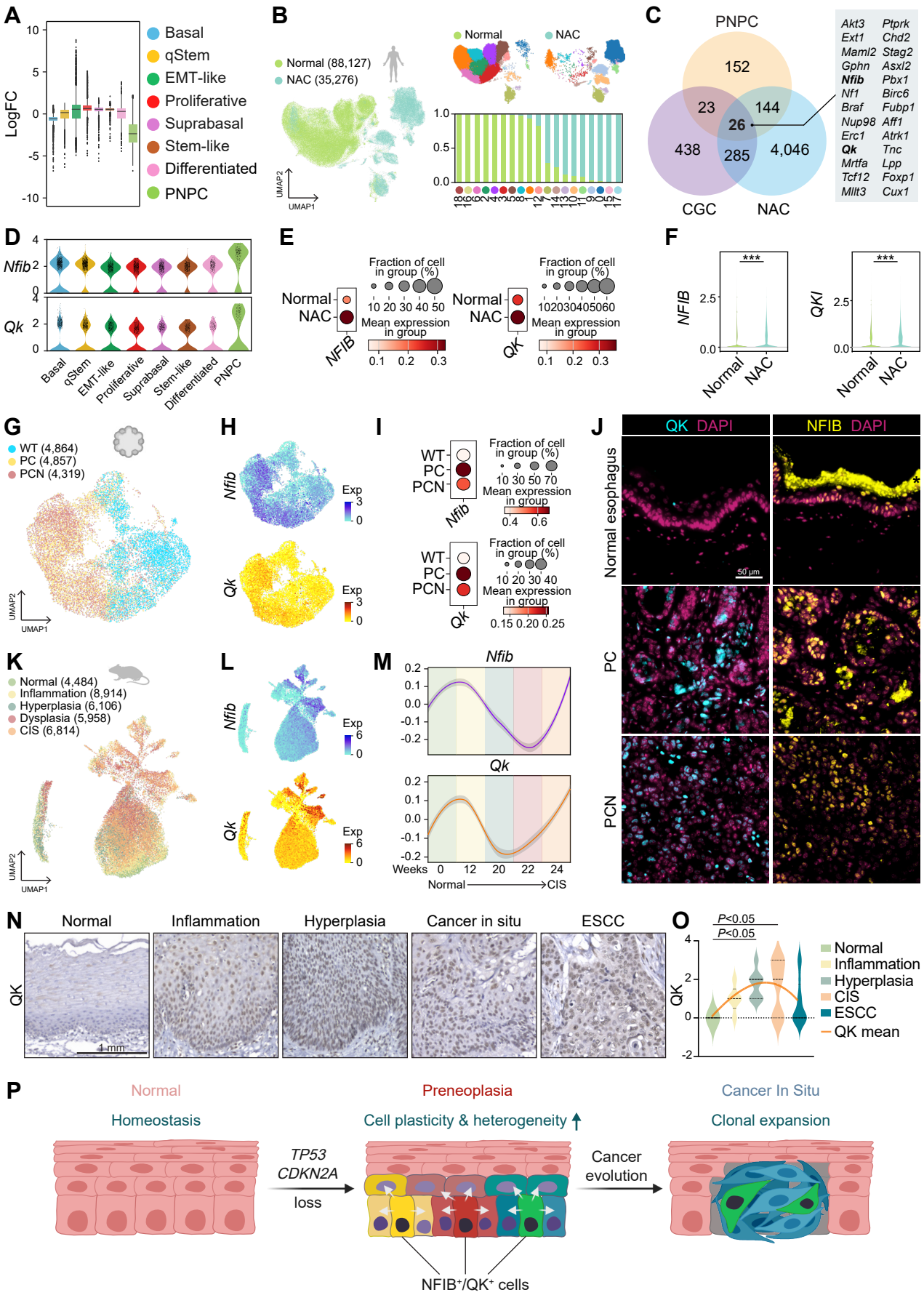
Front

Figure 4


A
LogFC
Basal
qStem
EMT-like
Proliferative
Suprabasal
Stem-like
Differentiated
PNPC
B
Normal (88,127)
NAC (35,276)
Normal
NAC
UMAP2
UMAP1
C
PNPC
152
23
144
26
438
285
4,046
CGC
NAC
Akt3 Ptprk
Ext1 Chd2
Maml2 Stag2
Gphn Asxl2
Nfib Pbx1
Nf1 Birc6
Braf Fubp1
Nup98 Aff1
Erc1 Atrk1
Qk Tnc
Mrtfa Lpp
Tcf12 Foxp1
Mllt3 Cux1
D
Nfib
Qk
Basal
qStem
EMT-like
Proliferative
Suprabasal
Stem-like
Differentiated
PNPC
E
Fraction of cell in group (%)
Normal
NAC
Mean expression in group
NFIB
Qk
F
NFIB
QKI
Normal
NAC
G
WT (4,864)
PC (4,857)
PCN (4,319)
H
Nfib
Qk
Exp
I
WT
PC
PCN
Fraction of cell in group (%)
Mean expression in group
J
QK DAPI
NFIB DAPI
Normal esophagus
PC
PCN
50 μm
K
Normal (4,484)
Inflammation (8,914)
Hyperplasia (6,106)
Dysplasia (5,958)
CIS (6,814)
L
M
Weeks
Normal
CIS
N
Normal
Inflammation
Hyperplasia
Cancer in situ
ESCC
QK
4 mm
O
P<0.05
P<0.05
QK
Normal
Inflammation
Hyperplasia
CIS
ESCC
QK mean
P
Normal
Homeostasis
Preneoplasia
Cell plasticity & heterogeneity
Cancer In Situ
Clonal expansion
TP53
CDKN2A
loss
Cancer evolution
NFIB+/QK+ cells