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Single-cell transcriptomics reveals long noncoding RNAs associated with tumor biology and the microenvironment in pancreatic cancer

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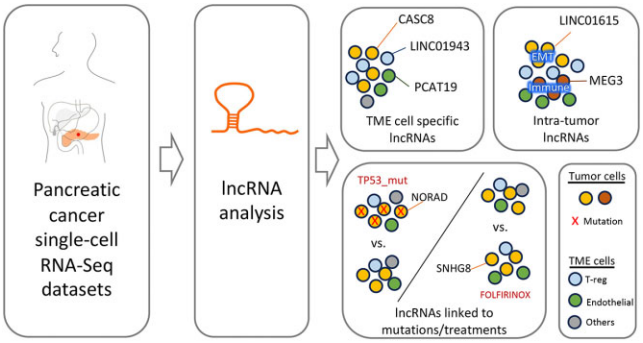
Abstract

Pancreatic ductal adenocarcinoma (PDAC) is highly heterogeneous and lethal. Long noncoding RNAs (lncRNAs) are an important class of genes regulating tumorigenesis and progression. Prior bulk transcriptomic studies in PDAC have revealed the dysregulation of lncRNAs but lack single-cell resolution to distinguish lncRNAs in tumor-intrinsic biology and the tumor microenvironment (TME). We analyzed single-cell transcriptome data from 73 multiregion samples in 21 PDAC patients to evaluate lncRNAs associated with intratumoral heterogeneity and the TME in PDAC. We found 111 cell-specific lncRNAs that reflected tumor, immune and stromal cell contributions, associated with outcomes, and validated across orthogonal datasets. Single-cell analysis of tumor cells revealed lncRNAs associated with *TP53* mutations and FOLFIRINOX treatment that were obscured in bulk tumor analysis. Lastly, tumor subcluster analysis revealed widespread intratumor heterogeneity and intratumoral lncRNAs associated with cancer hallmarks and tumor processes such as angiogenesis, epithelial–mesenchymal transition, metabolism and immune signaling. Intratumoral subclusters and lncRNAs were validated across six datasets and showed clinically relevant associations with patient outcomes. Our study provides the first comprehensive assessment of the lncRNA landscape in PDAC using single-cell transcriptomic data and can serve as a resource, PDACLncDB (accessible at https://www.maherlab.com/pdaclncdb-overview), to guide future functional studies.

Graphical abstract



Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest cancer types and is commonly diagnosed at advanced stages in ∼50% of patients (1). Despite research efforts toward this disease, little progress has been accomplished with respect to treatments for PDAC beyond combinations of cytotoxic chemotherapy. Currently, the median survival of PDAC is 6 months with a 5-year survival rate of ∼10% (1). The incidence of PDAC is increasing and is projected to be the second leading cause of cancer death by 2030 (2).

A major obstacle in the treatment of PDAC is the complex landscape of intra- and intertumor heterogeneity and the

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stroma-rich tumor microenvironment (TME). This high level of heterogeneity in PDAC provides opportunities for tumor evolution and progression during treatment, ultimately resulting in resistant disease. Recent genomic studies have highlighted the complex heterogeneity and evolutionary landscape in PDAC and the associated genomic alterations (3–5). However, due to the desmoplastic nature of PDAC tumors with the often sparse tumor cell populations, bulk sequencing efforts have been challenging to rigorously characterize PDAC tumors.

Advances in high-throughput single-cell sequencing have allowed us to begin to understand the contribution of the TME and its diverse cell types to cancer progression, tumor cell diversification and response to treatment (6–13). Consequently, this has led to a better distinction between tumor-intrinsic and -extrinsic characteristics such as the recent refinement of the classical and basal models as the major tumor-intrinsic subtyping in PDAC (13,14). Moreover, the heterogeneity of these multiple subtypes has been demonstrated intratumorally and throughout tumor progression (14). As such, PDAC tumor heterogeneity can also be driven by interactions between cancer cells and their surrounding TME resulting in changes in cancer cell states, plasticity and responses to therapies (15). Therefore, an understanding of tumor heterogeneity and the TME is crucial toward a better understanding of disease progression in PDAC.

Long noncoding RNAs (lncRNAs) are an important class of transcripts that play major roles in regulating tumor processes. The contributions of lncRNAs to PDAC tumor progression and response to treatments have recently been recognized (16). These include examples such as *ANRIL* and *MEG8*, which are activators of epithelial–mesenchymal transition (EMT), *CCAT1/2*, which promote PDAC cell proliferation, and *H19* in cell invasion and migration (16). Additionally, lncRNAs are known to exhibit strong cell type specificity with respect to gene expression and therefore can partake in a wide array of functions between tumor cells and the TME (17). Hence, understanding the involvement of lncRNAs in tumor heterogeneity and the TME offers a unique opportunity to explore their diverse roles in PDAC.

However, prior lncRNA transcriptomic studies in PDAC have relied primarily on bulk RNA sequencing (RNA-seq), which represents an admixture of gene expression across cells, thereby posing challenges in identifying lncRNAs enriched in TME cell types and associated with tumor heterogeneity (3,18). Moreover, recent single-cell transcriptomic studies of PDAC investigating tumor heterogeneity and the TME have largely ignored lncRNAs and focused on the limited number of highly expressed protein-coding genes (6,13). To address these limitations, we performed an lncRNA-focused analysis utilizing single-cell transcriptomics of PDAC patients in multiple independent cohorts and identified lncRNAs associated with the PDAC TME, intratumoral heterogeneity, mutational status, treatment and patient prognosis. LncRNAs identified in this analysis are available at the PDACLncDB resource: https://www.maherlab.com/pdaclncdb-overview.

Materials and methods

Single-cell RNA-seq datasets and clinical cohort

To evaluate the expression and characterize lncRNA-associated tumor-intrinsic biology, heterogeneity and the TME in PDAC, we utilized single-cell RNA-seq (10x Genomics, 3′ library) of 73 multiregion samples from the WUSTL (Washington University in St Louis) cohort of 21 patients with advanced PDAC, including treatment-naïve and those treated with chemotherapies such as FOLFIRINOX (6). Additionally, we utilized six independent but small cohorts for validation (72 samples; 26 561 cells) (7–13).

Preprocessing of single-cell RNA-seq for lncRNA analysis

The single-cell gene expression matrix and cell type classification were obtained from the original study (6) and used for cell type-specific lncRNA analysis. For tumor cell analysis, we performed additional preprocessing to account for the lower levels of expression and rate of detection for lncRNAs in individual tumor cells. These cells were often found to have high ribosomal RNA content. We determined a cutoff for eliminating these cells upon manual investigation of the distribution of the rate of undetectable genes and percent of ribosomal RNA.

Identification of TME cell type-specific lncRNAs in single-cell RNA-seq

TME cell type-specific lncRNAs were identified via differential expression analysis comparing cell types of interest and all other cell types using the marker identification function FindMarkers of the Seurat package (19). Cell specificity of lncRNAs was defined based on lncRNAs that were found to have significantly higher expression in a cell type compared to all other cell types [false discovery rate (FDR) < 0.001, log fold change (FC) > 1, detectable in $>25\%$ cells]. These genes were identified as markers of the corresponding cell types. To further evaluate the potential association of TME cell type-specific lncRNAs with patient outcome, survival analysis was performed using PDAC TCGA (The Cancer Genome Atlas) RNA-seq and clinical data ($n = 186$) (3), where lncRNA expression was fit to a Cox proportional hazard linear regression model for disease-free interval (DFI), disease-free survival, progression-free interval (PFI) and overall survival (OS). FDR was estimated using the Benjamini–Hochberg procedure.

Identification of lncRNAs associated with mutational status and FOLFIRINOX treatment

To identify lncRNAs associated with mutational status of *KRAS* or *TP53*, or treatment status, a differential expression analysis was performed confining to only the tumor cells from single-cell RNA-seq data (27 477 cells from 73 samples). The FindMarkers function in the Seurat package (19) was used to compare lncRNA expression between tumor cells in samples with mutation and those in samples harboring wild type (or between FOLFIRINOX-treated and treatment-naïve samples in treatment association analysis). To compare with bulk RNA-seq data, a similar differential expression analysis was performed using edgeR (20) (WUSTL, $n = 84$; TCGA, $n = 186$). An FDR of 0.05 was used to identify differentially expressed genes with $\log_2 FC > 0$ as upregulated and $\log_2 FC < 0$ as downregulated. Additionally, lncRNAs in chromosome X that were associated with FOLFIRINOX were removed due to an imbalance in the representation of male and female samples with this treatment. LncRNAs associated with mutation or FOLFIRINOX treatment were grouped into those identified exclusively via single-cell sequencing data, bulk sequencing data or both.

To further dissect potential functional contribution of the associated lncRNAs, pathway analysis was performed by aggregating single-cell expression for each sample into pseudo-bulk data, followed by scoring activity of lncRNAs and hallmark pathway genes using the combined *z*-score approach (21). Pathways with the highest Pearson correlation coefficients (PCCs) to lncRNA signatures were used for annotation. Validation of activity of FOLFIRINOX-associated lncRNAs derived from single-cell analysis was performed by using RNA-seq data from PDAC primary tumors ($n = 10$) and organoids ($n = 10$) with half naïve and half treated with FOLFIRINOX as obtained from Farshadi et al. (22).

Evaluation of intratumor heterogeneity via analysis of tumor subclusters

We aimed to evaluate intratumor transcriptional heterogeneity of PDAC tumor cells using single-cell RNA-seq data via a tumor subcluster analysis at case level. For each individual patient, tumor cells identified using PDAC tumor markers were segregated and reprocessed using the Seurat pipeline (19). Cell cycle scoring was performed, and single-cell gene expression was renormalized using the SCTransform procedure, regressing out mitochondrial content, total read count and unique molecular identifier count, ribosomal content and cell cycle scores. Principal component analysis and clustering was subsequently performed to group tumor cells to distinct clusters of cells with similar expression profiles (tumor subclusters) (Supplementary Figure S1A-C).

Characterization of tumor subclusters and associated lncRNAs

Upon identifying case-level tumor subclusters, these tumor subclusters were subsequently analyzed using Seurat to identify genes that were significantly differentially expressed in each tumor subcluster compared to other tumor subclusters from the same patient. These genes, including both protein-coding genes and lncRNAs, were called tumor subcluster deregulated genes. To further understand biological processes altered during tumor cell diversification among these subclusters, we first identified genes that are frequently deregulated during tumor diversification (found in three patients) regardless of other clinical and pathological variables (Supplementary Figure S1D and E). Frequently deregulated genes were then clustered based on their levels of deregulation (i.e. FCs) across tumor subclusters to identify groups of genes that exhibited similar alteration patterns during intratumor diversification (denoted as intratumoral gene signatures). Further annotation of intratumoral gene signatures was performed using a gene set enrichment analysis (GSEA) to identify enriched pathways within each gene signature (both protein-coding and lncRNA genes), using ClusterProfiler (23) and the MSigDB database (24). Finally, lncRNAs within each intratumoral gene signature were characterized to have association with the enriched pathways representing the gene signature.

Evaluation of intratumoral lncRNAs' association with patient outcome

Association of genes enriched in PDAC tumor subclusters and survival outcomes was performed using TCGA data ($n = 175$) (3) for bulk RNA-seq and outcomes of disease-specific survival (DSS), OS, PFI and DFI. Expression of each intratumoral gene signatures was performed using GSEA, then categorizing samples with high or low expression using a median cut-off, testing for associations with survival outcomes using a chi-squared test, followed by FDR correction to correct for multiple hypothesis testing.

Validation analysis of TME lncRNA markers and intratumoral gene signatures

To validate lncRNAs associated with TME and intratumor subclusters, single-cell RNA-seq data from (13), a re-analysis of six PDAC studies of single-cell transcriptomes was used. For TME-associated lncRNAs, 88 lncRNAs specific to cell types identified in the WUSTL discovery cohort were queried in this validation dataset for similarities in cell-specific expression after averaging expression values for each cell type using the Seurat package (13,19). We additionally used single-nucleus RNA-seq data from (25) to validate TME lncRNAs. For TME-associated lncRNAs, 33 lncRNAs specific to cell types identified in the WUSTL discovery cohort were queried in this validation dataset for similarities in cell-specific expression after averaging expression values for each cell type using the Seurat package (13,19).

To validate intratumoral lncRNA signatures, a tumor cell clustering was performed in the Chijimatsu *et al.* validation dataset to define tumor subclusters and their associated gene signatures. These signatures, which represented tumor heterogeneity in the validation dataset, were then matched with intratumor signatures in the discovery cohort via a correlation analysis. More specifically, marker genes for each PDAC tumor subcluster identified in the Chijimatsu *et al.* (validation) dataset were then correlated with intratumoral signatures discovered in the original (WUSTL) cohort after averaging gene expression for each patient (72 samples; 26 561 cells; Supplementary Figure S2A). Subsequently, each intratumoral gene signature of tumor subclusters in the WUSTL cohort was matched with a gene signature of tumor subcluster identified in the validation cohort with the maximum PCC. After assignments, permutation testing for 500 iterations was done to assess statistical significance by analyzing correlations with gene signatures of tumor subclusters in the WUSTL cohort and a random set of 500 genes. Empirical *P*-values were calculated as the fraction of permuted correlation coefficients greater than the observed correlation between assigned tumor subclusters, followed by FDR correction (Supplementary Figure S2B). UMAP (uniform manifold approximation and projection) plots for visualization were generated for gene sets using the AddModuleScore function in the Seurat package (19).

Results

Detection of lncRNA expression in tumor and TME cell types

Single-cell transcriptome sequencing allows for the quantification of gene expression at the individual cellular level. However, these data are often sparse with many undetectable genes (i.e. dropout), due to limited per-cell RNA quantity and sequencing depth (26). We evaluated and compared the detection levels for lncRNAs across different cell types identified in the TME of PDAC. For this analysis, we used PDAC single-cell transcriptome data from a recent publication of 73 multi-region samples from a cohort of 21 patients with PDAC (Figure 1A) (6). Of all annotated lncRNAs, 56% were expressed

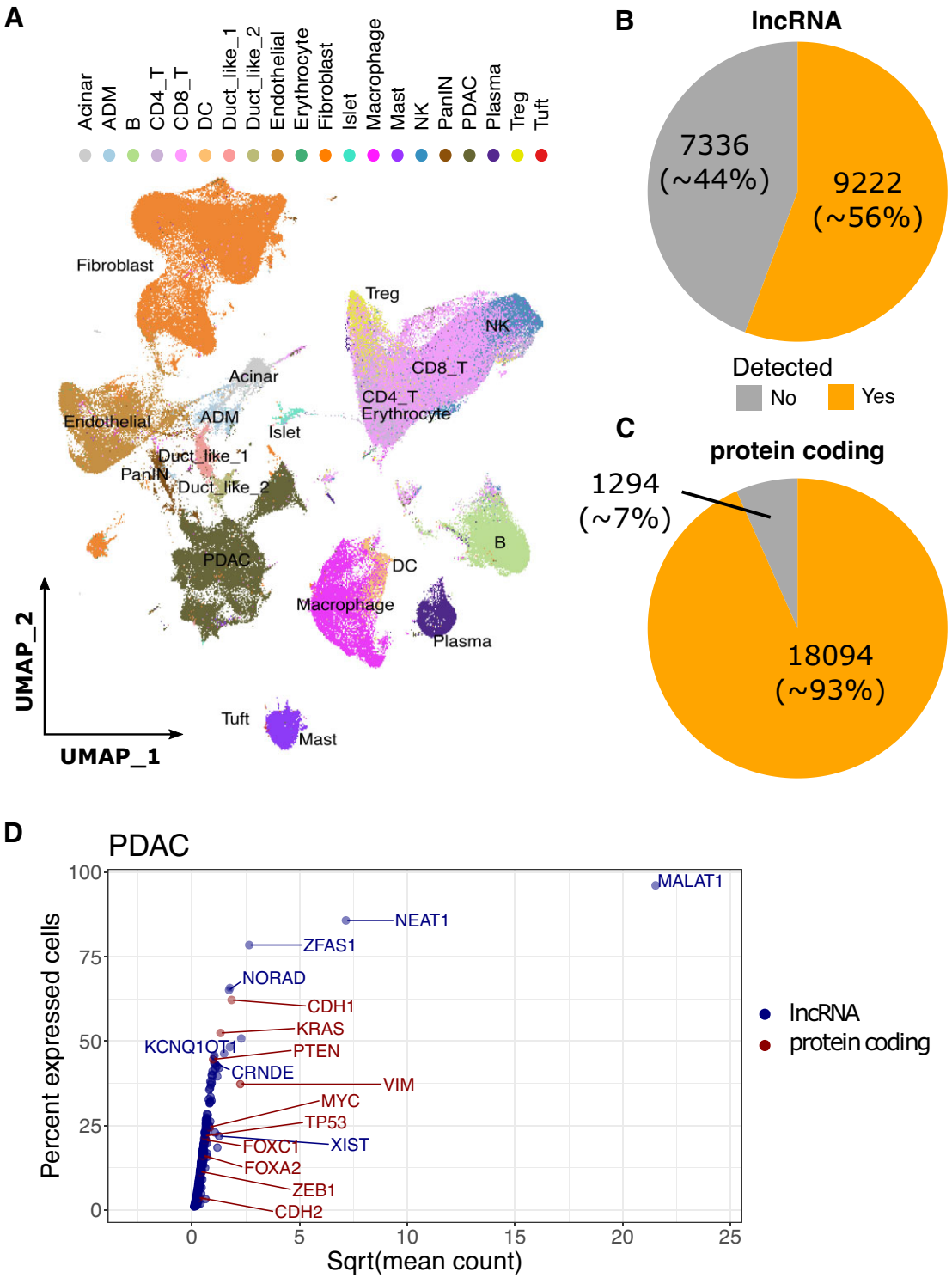

Figure 1. Comparison of rate of detection for lncRNA and protein-coding genes in single-cell transcriptome sequencing data in the WUSTL cohort. (**A**) UMAP plot of cell types identified in 73 tumor and adjacent normal samples from 21 PDAC patients. (**B**, **C**) Percentage of lncRNA and protein-coding genes detected in single-cell transcriptome sequencing data. (**D**) Percentage of PDAC tumor cells with detectable expression for lncRNAs and selected protein-coding cancer genes.

in this dataset across all cells compared to 93% of all annotated protein-coding genes (Figure 1B and C). Overall, the detection rate of lncRNAs was lower than that of protein-coding genes with most lncRNAs only detectable in <20% of cells and an average detection rate of 4.95% (compared to an average detection rate of 18% for protein-coding genes). As a result, an average of 1001 lncRNAs were detectable in >1% of cells with several lncRNAs detected at similar levels to protein-coding genes. These included lncRNAs known to be highly expressed, such as *MALAT1* and *NEAT1* (Figure 1D and Supplementary Figure S3A). We also observed that detection rates for lncRNAs varied by cell types with duct-like 1 cells showing the highest detection rate (6.3%) and acinar cells showing the lowest detection rate (3.4%) (Supplementary Figure S3A). Taken together, a significant number of lncRNAs were detectable at reliable levels for subsequent analyses.

LncRNAs associated with TME cell types

The contribution of the TME as a whole to cancer progression and response to treatments has recently been recognized (15). LncRNAs have also been shown to be involved in the TME in PDAC (27). To understand the landscape of lncRNAs in the PDAC TME, we first sought to quantify the levels of lncRNA expression throughout TME cell types. As expected, cell type marker analysis identified a large number of lncRNAs ($n = 111$) specific to a subset of TME cell types that were distinct from the original genes used to annotate cell types (Supplementary Table S1). Examples of known cell-specific lncRNAs that were detected include *SMIM25* expressed specifically in macrophages (28) and *LINC00926* expressed specifically in B cells (29) (Figure 2A). Among the top TME cell type-specific lncRNAs were *PCAT19*, which was predominantly expressed in endothelial cells, and *LINC01943* in regulatory T cells (Figure 2A). LncRNAs enriched in fibroblasts included *DNM3OS*, while those expressed specifically in PDAC tumor cells were *CASC8* and *CRNDE* (Figure 2A).

Next, we sought to validate cell-specific lncRNAs by using orthogonal datasets of PDAC single-cell transcriptome from recent publications (7–13). Of the lncRNA markers from the original WUSTL cohort, 88/111 lncRNAs exhibited expression in the validation cohorts. Several cell types had a high degree of overlap for cell-specific lncRNAs in the validation datasets, such as lncRNAs assigned to PDAC cells (6/8), T cells (9/14), B cells (5/6), macrophages (4/4) and fibroblasts (6/9), with less overlap for lncRNAs corresponding to endothelial cells (2/6), normal ductal cells (7/21) and islet cells (1/2). We found similarities in cell specificities for many individual lncRNAs, including *PCAT19* and *LINC01943* (Figure 2B). The lncRNAs enriched in PDAC cells, such as *CASC8* and *CRNDE*, were also highly expressed in ductal type 2 cells in the validation cohort as these are the corresponding malignant cell population in this dataset (Supplementary Figure S4A) Moreover, many lncRNAs specific to fibroblasts, such as *DNM3OS* and *DIO3OS*, were also expressed in fibroblasts in the validation cohort (Supplementary Figure S4A).

Furthermore, we have analyzed data in (25), which profiled 42 primary PDAC tumors using single-nucleus RNA-seq. This dataset only profiled the expression of 33/111 TME lncRNAs. However, we observed high level of cell-specific concordance in comparison with the WUSTL cohort (Supplementary Figure S4B and Supplementary Table S2). For example, validated TME lncRNAs included genes such as PDAC lncRNAs CASC8 and CRNDE, endothelial lncRNA PCAT19 and fibroblast lncRNA DIO3OS. Hence, the validation of these lncRNAs across different sequencing platforms and datasets highlights the cell-specific nature of these genes.

Lastly, to evaluate the potential clinical significance of cell type-specific lncRNAs, we performed survival analysis using bulk RNA-seq data from the TCGA PDAC cohort (3). Interestingly, we found that 21 tumor and TME cell type-specific lncRNAs were associated with patient outcome (Figure 2A). This included the tumor-specific lncRNA *CASC8* and the regulatory T cell-specific *LINC01943*, which are both associated with poor survival (Figure 2C–E). The level of expression of these lncRNAs likely corresponded to the proportion and activity of their specific TME lineages. Consequently, this result is consistent with earlier studies suggesting that high tumor burden or increased regulatory T-cell activity contribute to tumor progression and are associated with poor survival (30,31).

LncRNAs associated with mutational status

We next wanted to evaluate whether single-cell transcriptome sequencing could enable better identification of lncRNAs associated with mutational status of *KRAS* or *TP53*, which are the most frequently mutated genes in PDAC (3). For each gene, we performed lncRNA differential expression analysis comparing PDAC tumor cells between mutant and wild-type patients.

Our analysis of *TP53* mutational status revealed 245 differentially expressed lncRNAs, including *NORAD*, *NEAT1* and *MALAT1*, which have been shown to be associated with *TP53* mutations in prior reports (32) (Figure 3A and Supplementary Table S3). Next, we performed pathway analysis of these *TP53* mutant-associated lncRNAs by correlating lncRNAs with predefined signatures related to various biological processes. This demonstrated a correlation with genes implicated in KRAS, Notch and mitotic signaling (Figure 3B) and was similar to pathway analysis performed using protein-coding genes (Supplementary Figure S5A).

Next, a similar analysis was performed using bulk RNA-seq data from the same patient cohort. We found a large discrepancy between *TP53* mutant-associated lncRNAs identified using single-cell data and bulk RNA-seq data. Among genes expressed in both single-cell and bulk RNA-seq, 233 lncRNAs were found to be associated with *TP53* mutations in only single-cell data, 140 lncRNAs in only bulk data and 12 lncRNAs as detected by both datasets (Figure 3A). LncRNAs associated with *TP53* mutation in single-cell data but not in bulk data tended to be expressed in many TME cell types in addition to tumor cells such as the established *TP53*-associated lncRNAs *NORAD*, *NEAT1* and *MALAT1* (Figure 3C) (32). This suggests that single-cell data enable the delineation of expression in tumor cells from TME cells, which is obscured with bulk sequencing data. LncRNAs found to be associated with *TP53* mutation in bulk data only often showed low expression that is likely not detectable in single-cell data. Lastly, lncRNAs identified to be associated with *TP53* mutations in

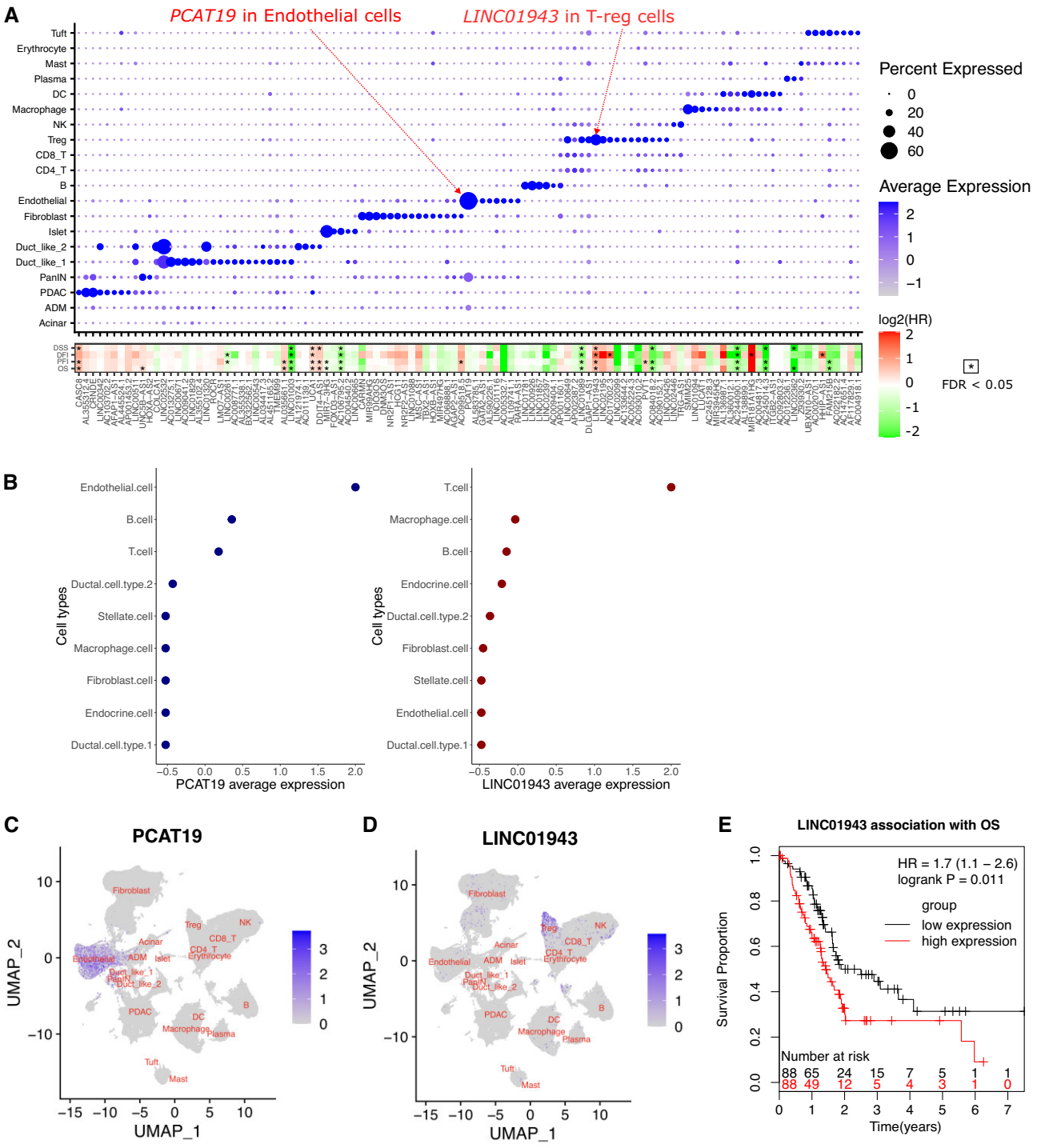

Figure 2. LncRNA markers of TME cells in PDAC. (**A**) Expression of 111 cell-specific lncRNAs in PDAC tumor and TME cell types identified in the WUSTL cohort (top) and their association with patient outcomes (bottom). (**B**) Average expression of lncRNAs *PCAT19* and *LINC01943* in cell types in validation cohorts. (**C**, **D**) Expression of representative cell-specific lncRNAs (*PCAT19* specific to endothelial cells; *LINC01943* specific to regulatory T cells) in the WUSTL cohort. (**E**) Association of *LINC01943* expression with patient OS (TCGA bulk RNA-seq).

both single-cell and bulk data included the lncRNA *CASC8*, a tumor-specific lncRNA that has been shown to be associated with p53 signaling in PDAC (33) and was also identified via a similar analysis of TCGA PDAC data (Supplementary Figure S5B). These findings illustrate the ability of single-cell transcriptome data to refine analysis performed using bulk sequencing data and allow for the identification of PDAC cell-specific changes in lncRNAs associated with *TP53* mutations.

In a similar analysis with *KRAS* mutation, we identified 47 lncRNAs associated with *KRAS* mutation (Supplementary Figure S5C and Supplementary Table S4). As most patients harbored *KRAS* mutation that also co-occurred with *TP53* mutation, many of the lncRNAs associated with KRAS mutation also associated with *TP53* mutation such as *NORAD*. Pathway analysis identified common upregulated pathways with *TP53* mutations, such as Notch and mitotic signaling, as correlated with *KRAS* mutations (Supplementary Figure S5C).

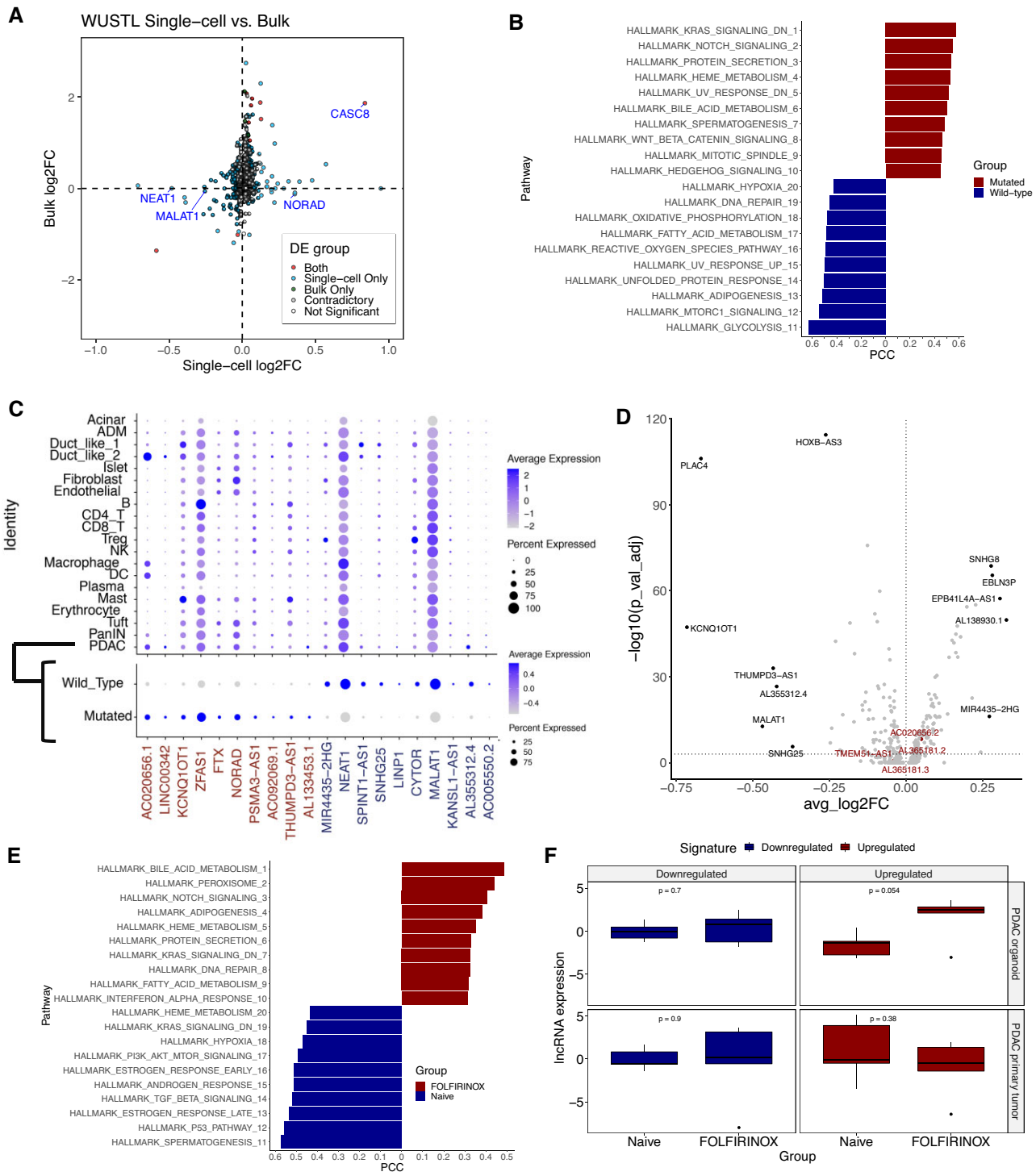

Figure 3. LncRNAs associated with TP53 mutations and FOLFIRINOX treatment. (**A**) Scatter plot of log FC for lncRNAs associated with TP53 mutations in single-cell sequencing (*x*-axis) and bulk sequencing in the WUSTL cohort (*y*-axis). LncRNAs with log FC values in both datasets are shown. LncRNAs are colored based on statistically significant association with TP53 mutation in single-cell data and/or bulk data. (**B**) Top 10 hallmark pathways correlated with the expression level of TP53 mutant-associated lncRNAs. (**C**) Expression at single-cell level of single-cell only TP53 mutant-associated lncRNAs. Top plot shows expression in PDAC and TME cell types. Bottom plot shows expression in mutated and wild-type PDAC cells. (**D**) Volcano plot of differentially expressed lncRNAs in FOLFIRINOX-treated PDAC cells. LncRNAs with $\log_2 FC > 0.5$ or $\log_2 FC < -0.5$ are labeled. Previously described lncRNAs are shown in red for comparison. (**E**) Top 10 hallmark pathways correlated with FOLFIRINOX lncRNAs. (**F**) Expression of FOLFIRINOX lncRNAs from PDAC organoids (O) and primary tumors (T) naïve ($n = 5$) and treated with FOLFIRINOX ($n = 5$) from the Farshadi *et al.* dataset. *P*-values from paired *t*-tests are listed above.

LncRNAs associated with FOLFIRINOX treatment status

Treatment with FOLFIRINOX is common in the neoadjuvant setting for patients with PDAC (34). However, lncRNAs in response to FOLFIRINOX therapy have not been well established. We performed differential expression analysis of PDAC tumor cells derived from single-cell transcriptomic data between patients who received FOLFIRINOX and those naïve to treatment. Due to an imbalance in the representation of male and female patients in this analysis, we chose to filter out lncRNAs on the X chromosome. Nevertheless, we identified several lncRNAs differentially expressed in tumor cells after treatment, such as *SNHG8* and *EBLN3P* (Figure 3D and Supplementary Table S5). Subsequent pathway analysis of lncRNAs derived from single-cell analysis identified metabolic and DNA repair pathways as upregulated in FOLFIRINOX-treated cells, which have been implicated in prior work focusing on protein-coding genes (22) (Figure 3E). We subsequently performed a similar analysis using bulk RNA-seq data from the same patient cohort and identified 36 differentially expressed genes via both methods (Supplementary Figure S5D). Common lncRNAs identified in both datasets included the lncRNA *SNHG8*, which has been identified in a prior report as associated with resistance to chemotherapy treatment in PDAC (35).

To validate our list of FOLFIRNOX-associated lncRNAs in an orthogonal dataset, we analyzed an independent patient cohort (36) and validated 67 FOLFIRINOX-associated lncRNAs (Supplementary Table S6). This includes lncRNAs upregulated with FOLFIRINOX, such as SNHG7 (average $\log_2$FC = 0.22) and EPB41L4A-AS1 (average $\log_2$FC = 0.29), and downregulated lncRNAs such as KCNQ1OT1 (average $\log_2$FC = −0.42) and PLAC4 (average $\log_2$FC = −0.25). Due to discrepancy and lack of cell type annotation, we were not able to validate all FOLFIRINOX-associated lncRNAs in this dataset. However, we further analyzed data from a prior report on patient-derived PDAC primary tumor samples and organoids isolated from patients who received ($n = 5$) or did not receive neoadjuvant therapy ($n = 5$) (22). We found that while lncRNAs derived using single-cell data that were identified as up- or downregulated during treatment in the original cohort did not show significant expression differences in PDAC primary tumors, there was a large expression increase for upregulated lncRNAs in PDAC organoids after FOLFIRINOX exposure (*t*-test *P*-value = 0.051) (Figure 3F). These results highlight the strong association between tumor cells and the set of therapy-associated lncRNAs derived via single-cell sequencing as these organoids are enriched for PDAC cells and depleted for components of the TME relative to primary tumors (22).

In summary, our findings identify lncRNAs associated with FOLFIRINOX treatment in PDAC and demonstrate the utility of single-cell sequencing to nominate PDAC-derived changes in the lncRNA landscape during neoadjuvant therapy.

Identification of lncRNAs associated with intratumor heterogeneity

PDAC is among the most heterogeneous cancer types. Earlier studies have reconstructed substantial intratumor heterogeneity represented by tumor subclones with unique mutational signatures within a tumor (6). Single-cell transcriptome data allow for an opportunity to identify subgroups of tumor cells with distinct expression profiles (tumor subclusters). Here, we aimed to understand the contribution of lncRNAs to distinct tumor subclusters via tumor subcluster analysis by leveraging the original (WUSTL) dataset (6) for discovery and additional independent smaller datasets (7–13) for orthogonal validation. To achieve better representation of lncRNAs in the WUSTL dataset, we removed tumor cells with a low percentage of detectable genes and excessive ribosomal content, corrected for cell cycle signaling, renormalized and performed case-level clustering of tumor cells (see the 'Materials and Methods' section). Our reanalysis identified 99 tumor subclusters from 21 patients with 1–7 clusters (mean = 3) per patient (Supplementary Figure S1A). We observed substantial spatial heterogeneity in tumor from individual patients whose tumor regions often harbored different proportion cells representing the tumor subclusters identified in each patient (Supplementary Figure S6). This highlighted the importance of multiregion sequencing to mitigate sampling bias due to spatial heterogeneity.

Tumor subcluster marker analysis identified 9560 genes including 514 lncRNAs differentially expressed in at least one subcluster when compared with other subclusters from the same patient. Genes frequently deregulated in tumor subclusters (at least three patients) were then subsequently clustered based on their levels of deregulation (estimated as FC across subclusters). We identified eight commonly deregulated subsets of genes including 89 lncRNAs that were enriched in signatures of tumor progression and tumor-related biological processes, including immune responses, glycosylation, metabolism, angiogenesis and EMT (Figure 5A and Supplementary Table S7). These deregulated gene subsets defined eight groups of tumor subclusters, each composed of subclusters from multiple patients with no obvious shared clinicopathological features. This suggests that these subsets of genes were commonly deregulated and contribute to PDAC intratumoral heterogeneity. We herein defined these commonly deregulated subsets of genes as intratumoral gene signatures.

Intratumoral lncRNAs are associated with TME interactions and tumor-intrinsic cellular processes

We further characterize the pathways enriched in the intratumoral gene signatures using GSEA of the combined protein-coding and lncRNA genes. The largest subset of intratumoral genes deregulated across tumor subclusters was enriched in immune signatures (WUSTL gene signature 1) whose top enriched gene sets included tumor necrosis factor-α signaling, T-cell activation and T-cell interaction (Figure 4A). We found 24 lncRNAs associated with the immune gene signature including *MEG3*, which has been shown to repress regulatory T-cell differentiation and immune escape in esophageal cancer (37) and is associated with immune infiltrates in glioma (38). The lncRNA *KCNQ1OT1* was also clustered in this gene set and is known to promote immune evasion and malignant progression in prostate and colorectal cancers (39,40). Lastly, the lncRNA *MALAT1* in this gene set has also been implicated in mechanisms related to immune evasion in pancreatic cancer (41).

The second largest subset of intratumoral genes was enriched for metabolism and glycosylation pathways (WUSTL gene signature 2) whose top enriched signatures included alcohol metabolism and O-linked glycosylation. The deregulated

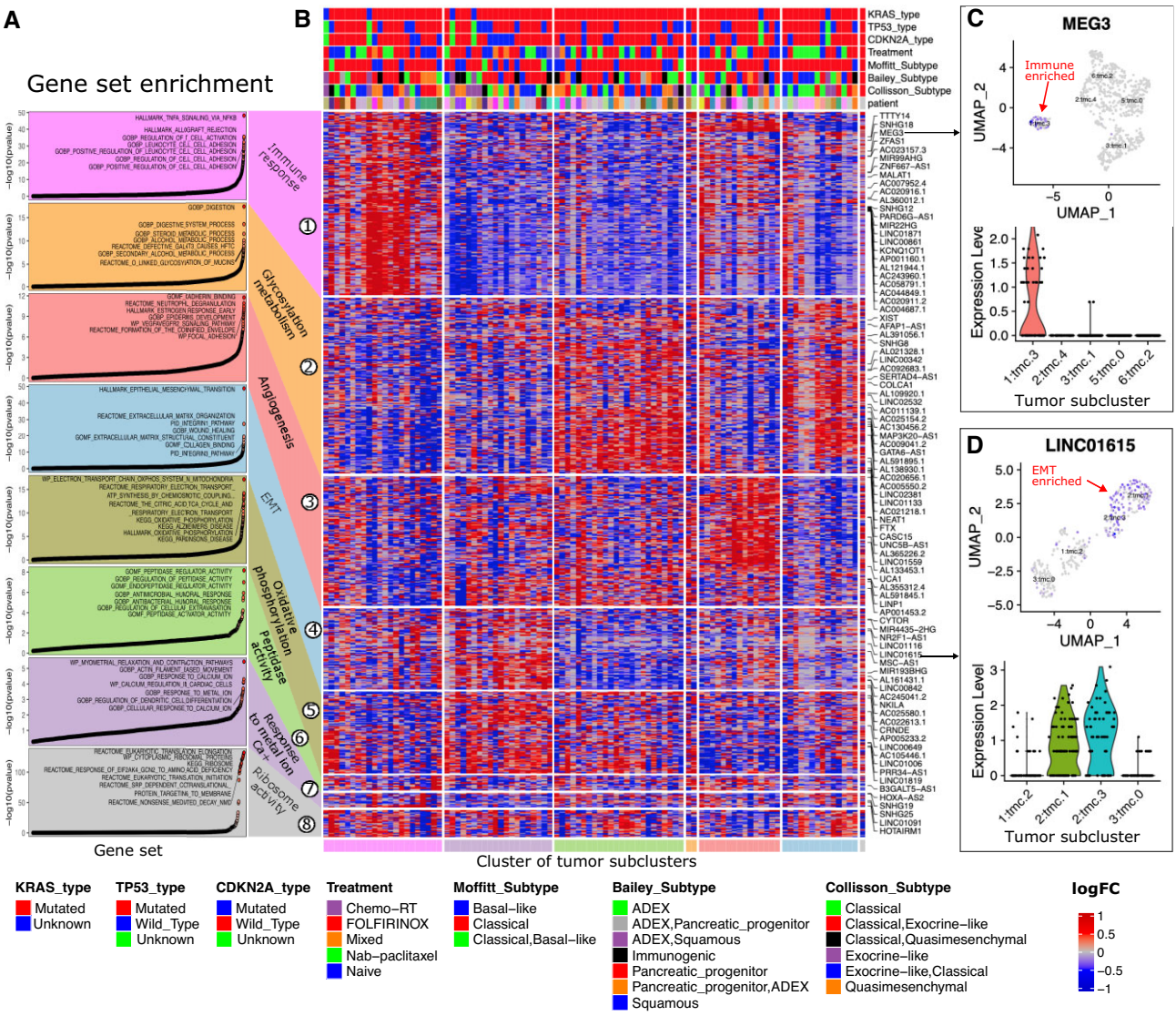

Figure 4. LncRNAs associated with PDAC intratumor heterogeneity and diversification. Shown is the characterization of eight gene clusters (protein-coding and lncRNA genes) identified to commonly deregulate in case-level tumor subclusters from the WUSTL cohort. (**A**) Gene set enrichment of individual gene clusters and summarization. (**B**) Heatmap representing the level of deregulation of individual genes in each tumor subcluster (defined as log FC in comparison with other tumor subclusters from the same patient). Only gene symbols for lncRNAs are shown (right). (**C**) Expression of MEG3, a representative lncRNA enriched in tumor subclusters associated with immune response. (**D**) Expression of LINC01615, a representative lncRNA enriched in tumor subclusters associated with EMT.

genes in this subset included 26 lncRNAs, among which were several lncRNAs known to promote tumor progression such as *XIST* and *COLCA1* (16,42). Additionally, we also identified lncRNA *FTX*, which is known to promote aerobic glycolysis and tumor progression in hepatocellular carcinoma (43).

The third largest subset of intratumoral genes was enriched for signatures of angiogenesis (WUSTL gene signature 3) whose top-ranked gene sets were cadherin adhesion and vascular endothelial growth factor signaling pathways. We identified 10 lncRNAs associated within the angiogenesis subset including *UCA1*, which was previously shown to promote angiogenesis in pancreatic cancer (44).

The fourth subset of deregulated intratumoral genes was enriched for gene signatures of EMT (WUSTL gene signature 4). We identified 12 lncRNAs associated with EMT including *NKILA*, which has been known to promote EMT in liver cancer (45), *LINC00842* (*ELIT-1*), a known activator of transforming growth factor-β (TGF-β)-mediated EMT (46), and *CRNDE*, which has been demonstrated to promote aggressive tumor phenotypes including EMT in multiple cancers (47).

Lastly, we found a subset of intratumoral deregulated genes enriched for the signature of oxidative phosphorylation (WUSTL gene signature 5), whose members included eight lncRNAs. Additionally, two small subsets of intratumoral genes were found to be associated with peptidase activity (WUSTL gene signature 6) and response to calcium (WUSTL gene signature 7), respectively. Several lncRNAs were included in these gene signatures, including *HOXA-AS2* (associated with peptidase activity) (48) and *HOTAIRM1* (associated with calcium handling) (49). In summation, we identified eight PDAC intratumoral gene signatures that are independent of clinicopathological features, with seven containing lncRNAs correlated with biologically relevant pathways representing tumor-intrinsic biology and interactions with the TME.

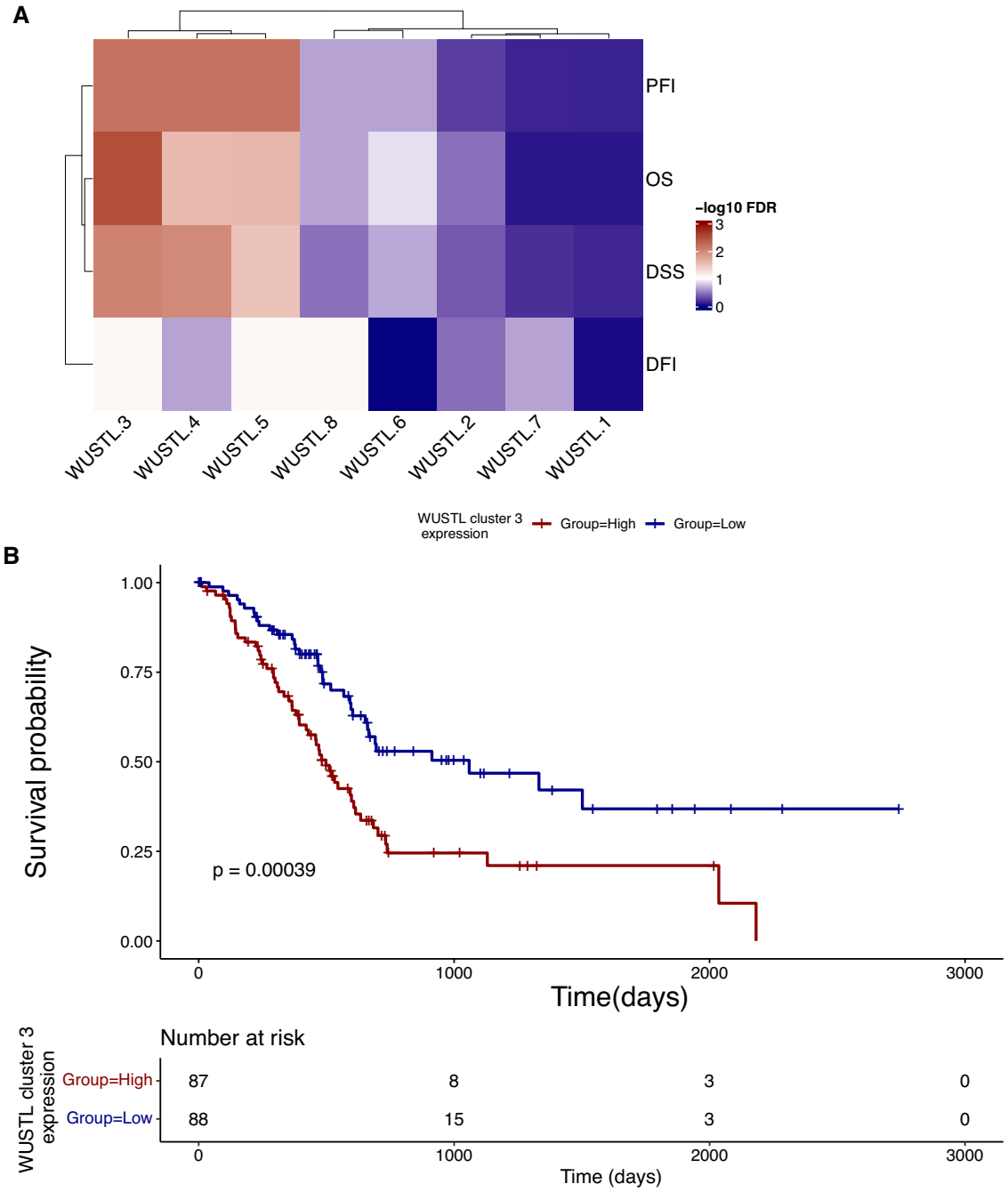

Figure 5. Association with patient outcome of PDAC intratumor gene clusters. (**A**) Heatmap of the associations (log_{10}FDR) between genes in the WUSTL intratumor gene cluster and PFI, OS, DSS and DFI from TCGA PAAD data ($n = 175$). (**B**) KM plot of OS of TCGA PAAD patients stratified by the GSEA score of WUSTL gene cluster 3 (angiogenesis).

PDAC intratumoral gene signatures are associated with patient outcomes

Next, we wanted to assess the clinical significance of lncRNAs associated with intratumoral heterogeneity. As prior work has delineated an association between subtypes of PDAC and outcomes (4), we sought to define any relationships between the intratumoral gene signatures identified in the WUSTL cohort with clinical outcomes using TCGA data (see the 'Materials and Methods' section). Analysis of intratumoral gene signatures and various survival outcomes found a statistically significant relationship between WUSTL intratumoral gene signatures 3 (angiogenesis), 4 (EMT) and 5 (oxidative phosphorylation), and DSS, OS and PFI in PDAC patients (Figure 5A). This suggests that a subset of tumor subclusters enriched for these signatures likely represent more aggressive tumor cell subpopulations. In fact, prior studies have shown that patients whose tumors are enriched for EMT markers demonstrated poor prognosis (4). Individual Kaplan–Meier (KM) curves further established this association between these tumor subclusters and poor prognosis (Figure 5B and Supplementary Figure S7). Hence, we identified clinically relevant associations between PDAC tumor subclusters and

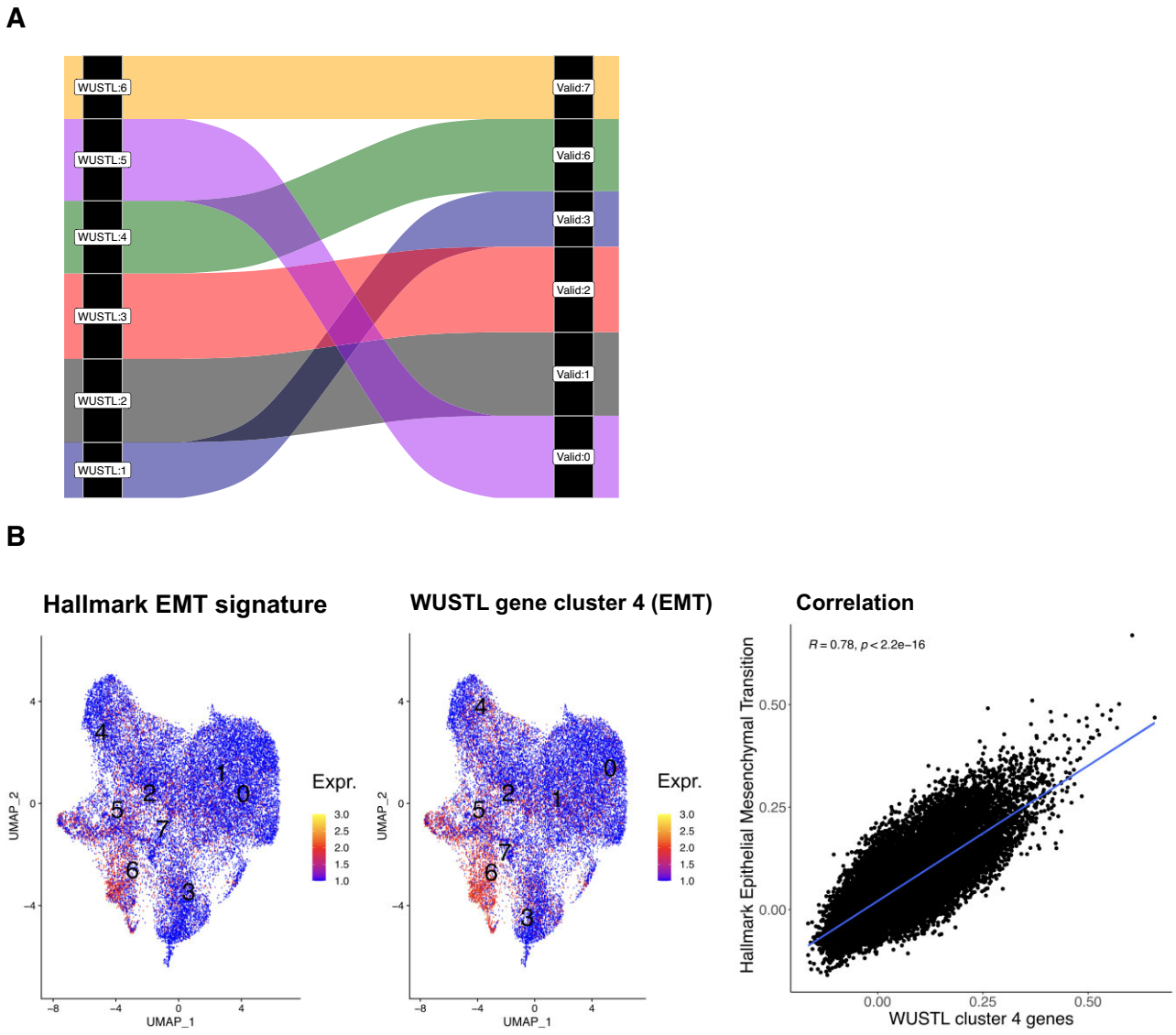

Figure 6. Evaluation of intratumoral lncRNAs and gene signatures in additional PDAC datasets. (**A**) River plots of the correlation between intratumoral gene clusters in the original dataset (WUSTL) and those identified in validation cohorts (Valid) exhibiting maximum PCC between marker genes and significant *P*-value after permutation testing. Width of connections is proportional to the PCC. (**B**) UMAP plot of activity of hallmark EMT signature genes (left) and WUSTL EMT gene cluster (cluster 4) (center), and correlation plot of the score for the two (right).

patient outcomes, highlighting the role for their corresponding lncRNAs in determining prognosis.

Orthogonal evidence of intratumoral lncRNAs and their associations with TME and tumor-intrinsic cellular processes

To further validate our findings of lncRNAs associated with intratumoral heterogeneity in PDAC, we used an orthogonal dataset consisting of six previously published single-cell transcriptomic analyses of PDAC (7–13). Tumor subcluster analysis was performed in the prior report and was used to query lncRNAs associated with tumor diversification. By analyzing the correlation of intratumoral genes in this orthogonal dataset, we were able to find that intratumoral gene signatures identified in the WUSTL cohort exhibited strong correlation and statistical significance with gene signatures of tumor subclusters in the validation (Valid) dataset (see the 'Materials and Methods' section) (Figure 6A and Supplementary Figure S2A and B).

As an example, the EMT gene signature (WUSTL gene signature 4) demonstrated a strong positive correlation (PCC = 0.75) with gene signatures of tumor subcluster 6 in the validation cohort (Valid cluster 6), which were also enriched for canonical EMT signaling genes (Figure 6B). In another example, the glycosylation–metabolism gene signature (WUSTL gene signature 2) exhibited the strongest association (PCC = 0.86) with gene signature of tumor subcluster 1 in the validation cohort (Valid cluster 1), which was enriched for metabolism-related signaling pathways and a robust correlation (Supplementary Figure S8A). Finally, the angiogenesis gene signature (WUSTL gene signature 3) showed positive correlation (PCC = 0.87) with the gene signature of tumor subcluster 2 in the validation cohort (Valid cluster 2) that was also enriched for cadherin signaling (Supplementary Figure S8B). In summary, our findings demonstrate that PDAC intratumor

heterogeneity and the associated intratumoral lncRNAs represent universal features of tumor progression and interactions with the TME.

Discussion

In this study, we presented a characterization of lncRNAs in PDAC TME and intratumor heterogeneity using single-cell transcriptomic data. While the level of expression of lncRNAs is typically lower than that of protein-coding genes, a significant number of lncRNAs were detectable in the single-cell RNA-seq data used in this study. This allowed us to identify novel lncRNA markers of TME cell types, as well as lncRNAs associated with intratumor heterogeneity and tumor-intrinsic processes.

The expression of lncRNAs was found to be highly cell type specific. Our analysis utilizing single-cell RNA-seq data revealed cell-specific lncRNA markers seen in orthogonal datasets, including those that have been characterized in the literature and can be queried using our resource PDACLncDB. For example, *PCAT19* was found to be enriched in endothelial cells, which has been implicated in regulating the switch from quiescence to proliferation in these cells upon vessel injury (50). As fibroblasts are known to be important components of the TME in pancreatic cancer, we identified several lncRNAs enriched in these cell types. These include *DNM3OS*, which has been demonstrated to participate in TGF-β signaling in lung fibroblasts and contribute to pulmonary fibrosis (51). In addition, the lncRNA *CRNDE* was identified as enriched in PDAC tumor cells, and studies have shown it to be upregulated in PDAC and associated with poor prognosis due to its role in cancer cell proliferation and apoptosis (47). Due to cell-specific expression, the level of TME-specific lncRNAs could represent the proportion and activity of the corresponding cell type. However, we also identified tumor-specific lncRNAs associated with patient outcome that were known to drive tumor progression, such as CASC8 and UCA1, suggesting the potential of our analysis to identify functionally significant lncRNAs.

The ability to establish the cellular identity of genes represents a major advantage of single-cell sequencing data compared with bulk sequencing data. This is exemplified by our discovery of tumor-intrinsic associations between lncRNA expression and *TP53* mutations using single-cell data that were obscured in bulk transcriptome data. For example, by confining our analysis to tumor cells in single-cell transcriptome, we identified the lncRNA *NORAD* and its association with *TP53* mutations. Earlier studies have showed that *NORAD* is a downstream target of *TP53* and acts as a tumor suppressor to maintain genome stability (32). This is consistent with our result showing *NORAD* upregulation in *TP53* mutant PDAC cells, suggesting an alternative mechanism of genome stability maintenance. Additionally, pathway analysis of lncRNAs that were correlated with *TP53* mutations included KRAS signaling, which has been described as an early mutation in pancreatic cancer progression (52), Notch signaling, which has been described to elicit crosstalk with TP53 signaling (53), and mitotic signaling, indicating a failure of cell cycle arrest that has been described as associated with these mutations (54). Results from both single-cell and bulk sequencing datasets showed a robust upregulation of the lncRNA *CASC8*, which is a PDAC-specific lncRNA and has been described as implicated in TP53 signaling and proliferative pathways in PDAC through an interaction with H19, miR-671 and SMAD7 (33).

Similar to mutational association analysis, our tumor-intrinsic analysis of FOLFIRINOX-associated lncRNAs revealed novel genes such as *SNHG8* to be increased in PDAC cells, which is consistent with reports of its role in chemosensitivity to gemcitabine in PDAC (35). However, lncRNAs identified in a prior report using bulk sequencing (22), such as *TMEM51-AS1*, *AC020656.2*, *AL365181.2* and *AL365181.3*, demonstrated relatively weaker differential expression. Correlated pathways with single cell-derived lncRNAs demonstrated an association with DNA repair and metabolic pathways. Prior work focusing on protein-coding genes in PDAC has shown a similar increase in DNA repair and alternative metabolism signaling genes upon FOLFIRINOX treatment (22). As a proof of concept, we showed the utility of single-cell analysis to separate FOLFIRINOX-treated from treatment-naïve PDAC organoids in a validation dataset based on lncRNA expression profiles. Future studies profiling lncRNAs from larger cohorts of PDAC cells exposed to FOLFIRINOX treatment will be needed to verify these findings. Collectively, these results highlight the advantage of single-cell analysis to delineate previously underappreciated tumor-intrinsic alterations of lncRNAs in response to *TP53* inactivation and FOLFIRINOX treatment that can be further explored mechanistically in functional studies using our resource.

Our final analysis of tumor subclusters revealed widespread intratumor heterogeneity and diversification based on consistent changes in PDAC tumor cells across patient samples and validated across various PDAC datasets. These mechanisms of heterogeneity involved altered activity of common oncogenic pathways, including immune responses and EMT. Through a joint analysis of protein-coding genes and lncRNAs, we were able to discover and associate lncRNAs commonly deregulated during intratumor diversification with various oncogenic pathways. These include the lncRNA *MEG3*, which was associated with genes involved in immune and inflammatory signaling. This is consistent with prior reports on the role of *MEG3* in esophageal cancer to decrease expression of *FOXP3* and subsequently regulatory T-cell differentiation and immune escape (37). Another lncRNA in this gene set that was uncovered was *MALAT1*, which has been associated with increased expression of PD-L1 expression in PDAC via *METTL3* (41). We identified a PDAC gene signature enriched for metabolism and glycosylation pathways, which included the lncRNA *FTX* and is consistent with reports of *FTX* and its role in regulating expression of carbohydrate metabolism genes such as *PPARγ*, leading to increased aerobic glycolysis in hepatocellular carcinoma (43). EMT signaling was also found to be enriched in a subset of PDAC tumor subclusters and associated with a subset of intratumoral lncRNAs. The lncRNAs *LINC01615* and *LINC00842* were both detected in this signature, similar to recent reports of their associations with EMT genes through SIPA1 and TGF-β signaling, respectively (46,55). Moreover, testing of these genes with survival outcomes in PDAC patients identified a statistically significant association between high EMT signaling and poor prognosis that was independent of clinicopathological features, suggesting a role for this biological pathway and its corresponding lncRNAs in driving aggressiveness in PDAC. This is similar to prior reports on quasi-mesenchymal subtypes of PDAC showing high expression of mesenchymal markers and associations with poor outcomes (4). In addition, other pathways

were found to be commonly associated with PDAC intratumor heterogeneity, including angiogenesis and oxidation phosphorylation, which both also demonstrated significant associations with patient survival. Prior literature has found a poor prognostic association between protein-coding genes associated with angiogenesis and PDAC outcomes (56,57), as well as data showing that oxidative phosphorylation drives PDAC cells toward a cancer stem cell phenotype, which is known to be associated with aggressive disease and poor prognosis (58,59). The lncRNAs associated with intratumor diversification collected in our resource can aid in further mechanistic and functional characterizations of their roles in diverse and clinically important tumor processes.

In summary, we utilized single-cell transcriptomic data from PDAC patients coupled with bulk transcriptomic, genomic and clinical data to comprehensively characterize the lncRNA landscape in this disease. In doing so, we identified lncRNAs associated with the TME, *TP53* mutations, FOLFIRINOX treatment and intratumoral heterogeneity. In addition, our findings highlight the utility of single-cell sequencing to ascribe lncRNA alterations to particular cell types and nominate potential mechanisms of action. These results contained in PDACLncDB will serve as a resource to inform future work on identifying the biological roles of these lncRNAs and their contributions to PDAC.

Data availability

The lncRNAs associated with the TME, TP53 mutations, FOLFIRINOX treatment and tumor subclusters are available at the PDACLncDB resource (https://www.maherlab.com/pdaclncdb-overview). All data analyzed during this study are included in the following published articles and their supplementary information files (3,6,13).

Supplementary Data

Supplementary Data are available at NAR Cancer Online.

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Conflict of interest statement

None declared.

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