

❖ Background & Methods

Lung cancer remains amongst the most commonly diagnosed cancer type ranking as the leading cause of cancer-related death in the United States. In the lung squamous cell carcinoma (LUSC) subtype, basal cells are considered to be the cell of origin with few identified driver mutations to date. However, chromosome or chromosome arm imbalance (aneuploidy) is present since the early development of LUSC. Alterations affecting chromosome 3 are extremely common in LUSC, where in stage I 80% and 60% of the cases (TCGA data) show loss of chromosome arm 3p and gain of chromosome arm 3q, respectively. These findings suggest a possible role for aneuploidy in tumor initiation. We hypothesize, that aneuploidy might contribute to disrupt the normal differentiation of basal cells as an early event in LUSC development. To study this phenomenon, our lab genetically engineered immortalized human lung epithelial cells to harbor either chromosome 3p deletion (3pDel) or chromosome 3q gain (3qGain) using CRISPR (Fig.1). We then induced differentiation in wild type (WT) and aneuploid clones grown in organoids and characterized phenotypes at the histological, morphological and gene expression level using bulk and scRNAseq.

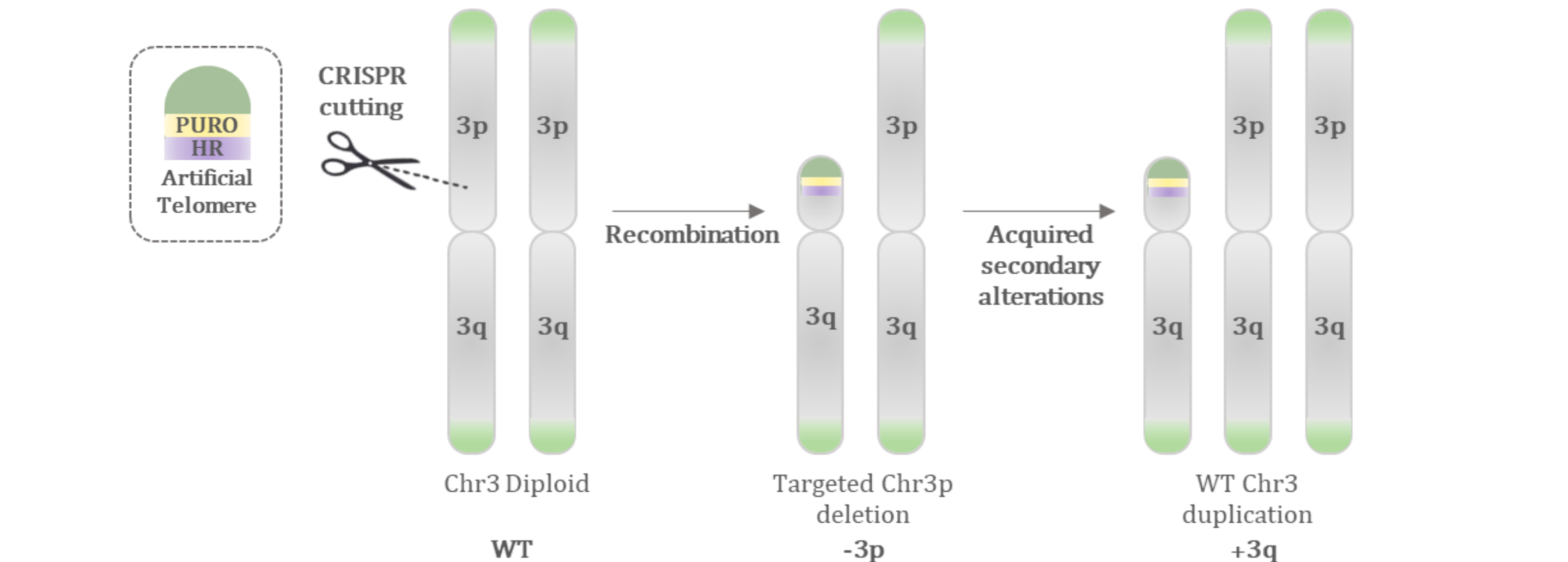


Fig 1. Schematic representation of the CRISPR-Cas9 approach employed to generate targeted chromosome 3p arm deletion in immortalized airway lung epithelial cells (AALEs).

❖ Results

1. Engineered AALEs are basal-like cells

Using RNAseq we compared the gene-expression pattern of our AALEs to an available GEO dataset containing basal and basal differentiated cells' gene expression information (GSE137557)(Fig.2A). This comparison together with the detection of the canonical basal cell markers TP63 and KRT5 by immunofluorescence (Fig.2B), allowed us to classify our AALEs as basal-like cells. Additionally, using a gene-signature derived from Travaglini et al 2020, we were able to identify the gene expression pattern of the aneuploid AALEs as basal proliferating, while WT counterparts showed a canonical basal cell gene expression pattern (Fig. 2C). After differentiation in 3D culture, both immortalized HBEC3 cells and WT AALEs showed squamous morphologies differing from the control pHBECS, indicating a differentiation disruption due to the immortalization background. However, WT AALE organoids showed well-structured basal layers populated with epithelial-shaped cells expressing KRT5, 13 and 14 in a similar fashion to pHBECS, but with different internal organization and absence of ciliated cells (Fig. 2D).

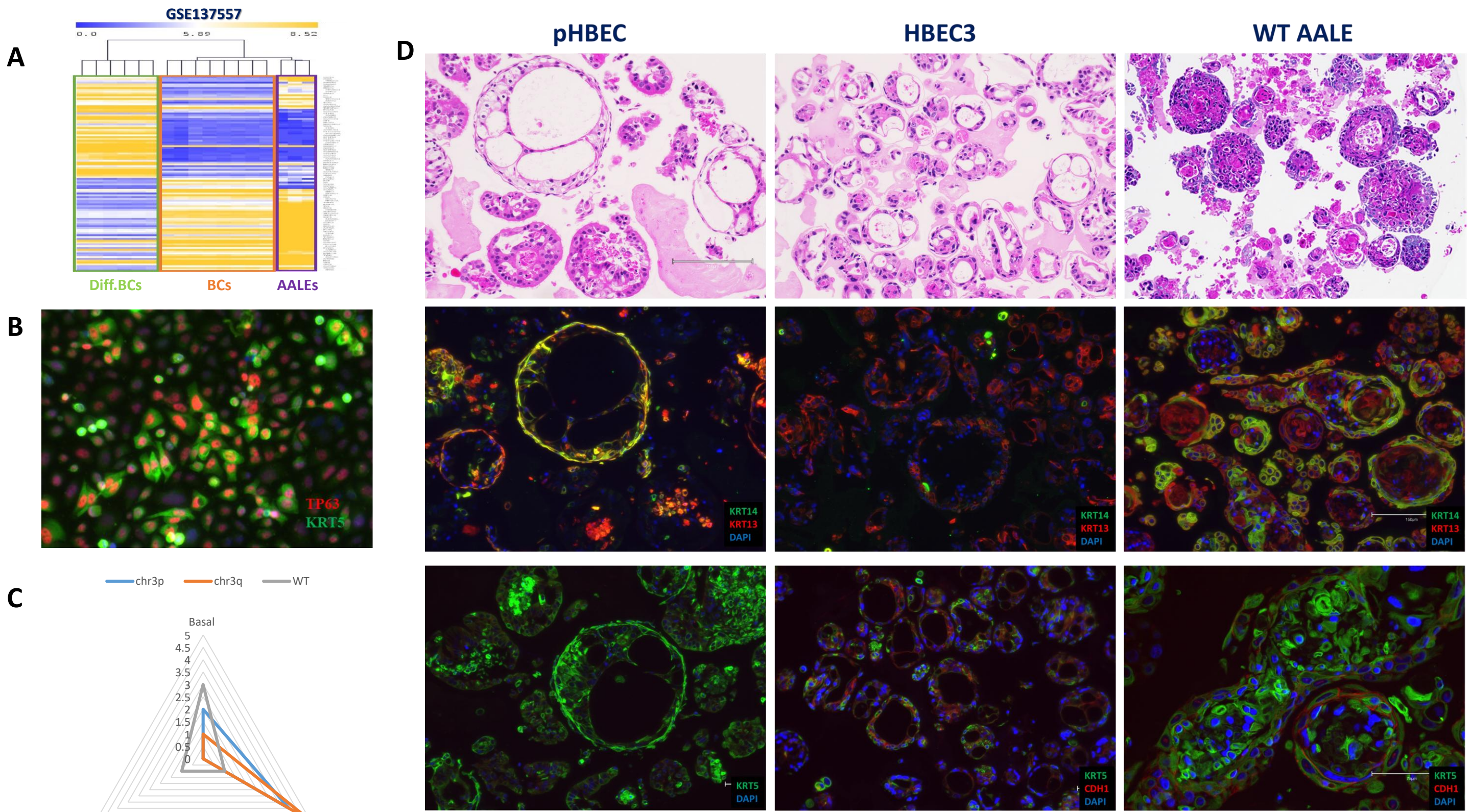


Fig 2. A) Hierarchical clustering comparing basal (green), basal differentiated (orange) and engineered AALEs (purple) gene expression patterns. **B)** AALEs immunofluorescence TP63 (red), KRT5 (green). **C)** Gene signature from Travaglini et al 2020 and radar plot for AALEs comparing WT, 3pDel and 3qGain genotypes. **D)** H&E staining and immunofluorescence for pHBECS, HBEC3 and WT AALE organoids after differentiation.

2. Histological analysis revealed genotype-specific phenotypes

After differentiation, both WT and aneuploid organoids showed squamous morphologies with noticeable genotype-related differences. WT organoids showed well-structured basal layers populated with epithelial-shaped cells, whereas aneuploid organoids presented unorganized basal layers with stretched cells. 3qGain organoids showed squamous carcinoma features such as the accumulation of keratin pearls. While, at the mRNA level, expression of canonical differentiation markers CC10 and MUC5B was induced in all genotypes (Fig. 3A); their expression was only detectable in WT organoids at the protein level. KRT14 expression was traceable to KRT14Low/KRT5High epithelial-shaped cells located in the basal layer of the WT organoids. In contrast, in 3qGain organoids, these cells showed to be KRT14High/KRT5Low with flattened morphologies located at the organoid's basal layer (Fig. 3B).

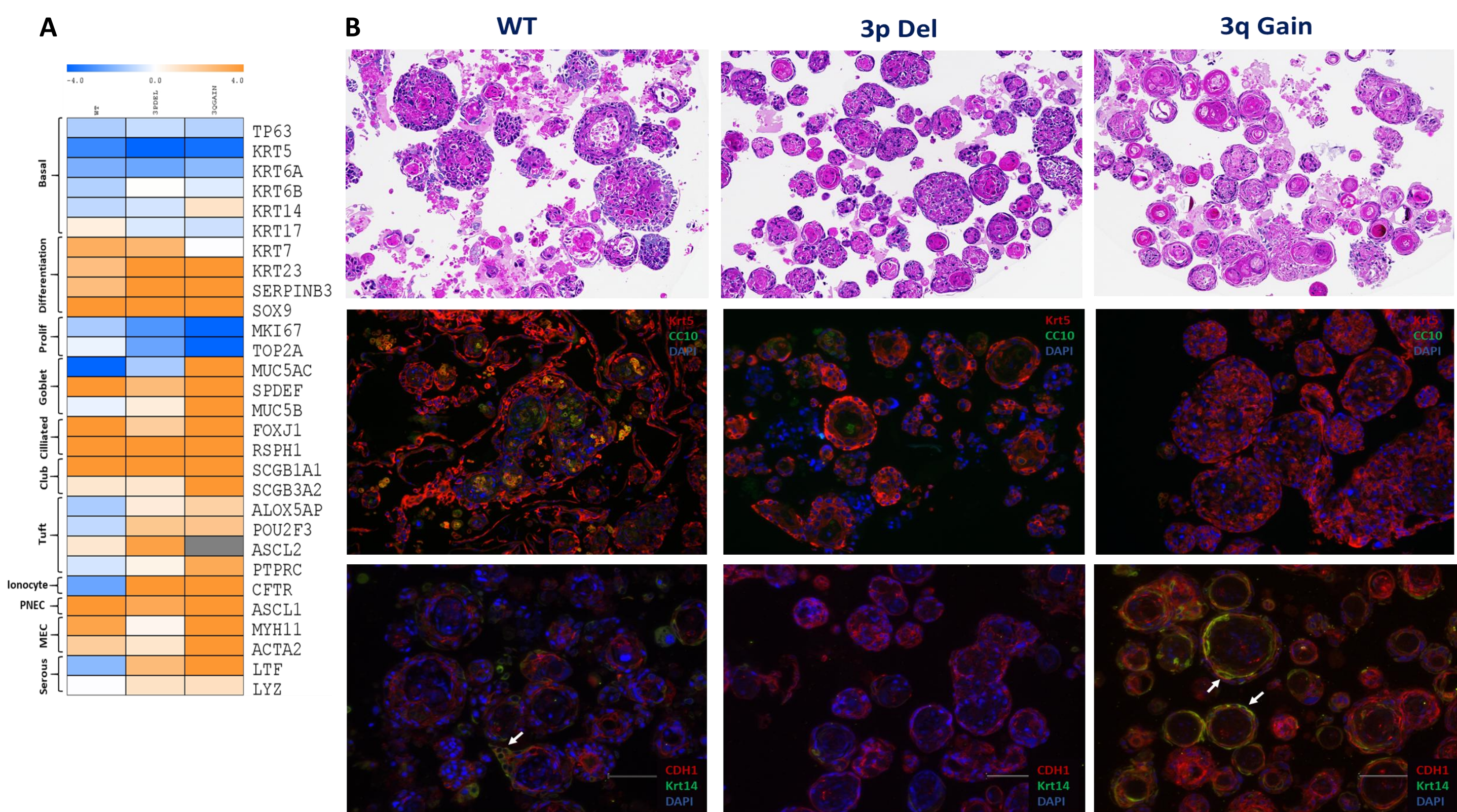


Fig 3. A) Gene expression FC after differentiation for AALEs organoids **B)** Representative images of WT, 3pDel and 3qGain organoids after differentiation. **Top)** H&E staining. **Mid)** Immunofluorescence KRT5 (red) and CC10 (green). **Bottom)** Immunofluorescence CDH1 (red) and KRT14 (green).

3. Differentiation generates genotype-specific gene expression patterns and cell populations

Bulk RNAseq in our organoids revealed specific gene expression patterns related to each of the genotypes (WT, 3pDel and 3qGain) as well as each of the studied timepoints. Interestingly, WT AALEs tend to cluster closer to pHBECS while 3pDel tend to cluster with HBEC3 (Fig. 4A). These results are in line with our scRNAseq observations that indicate that each genotype tend to generate separated clusters both before and after differentiation (Fig. 4B).

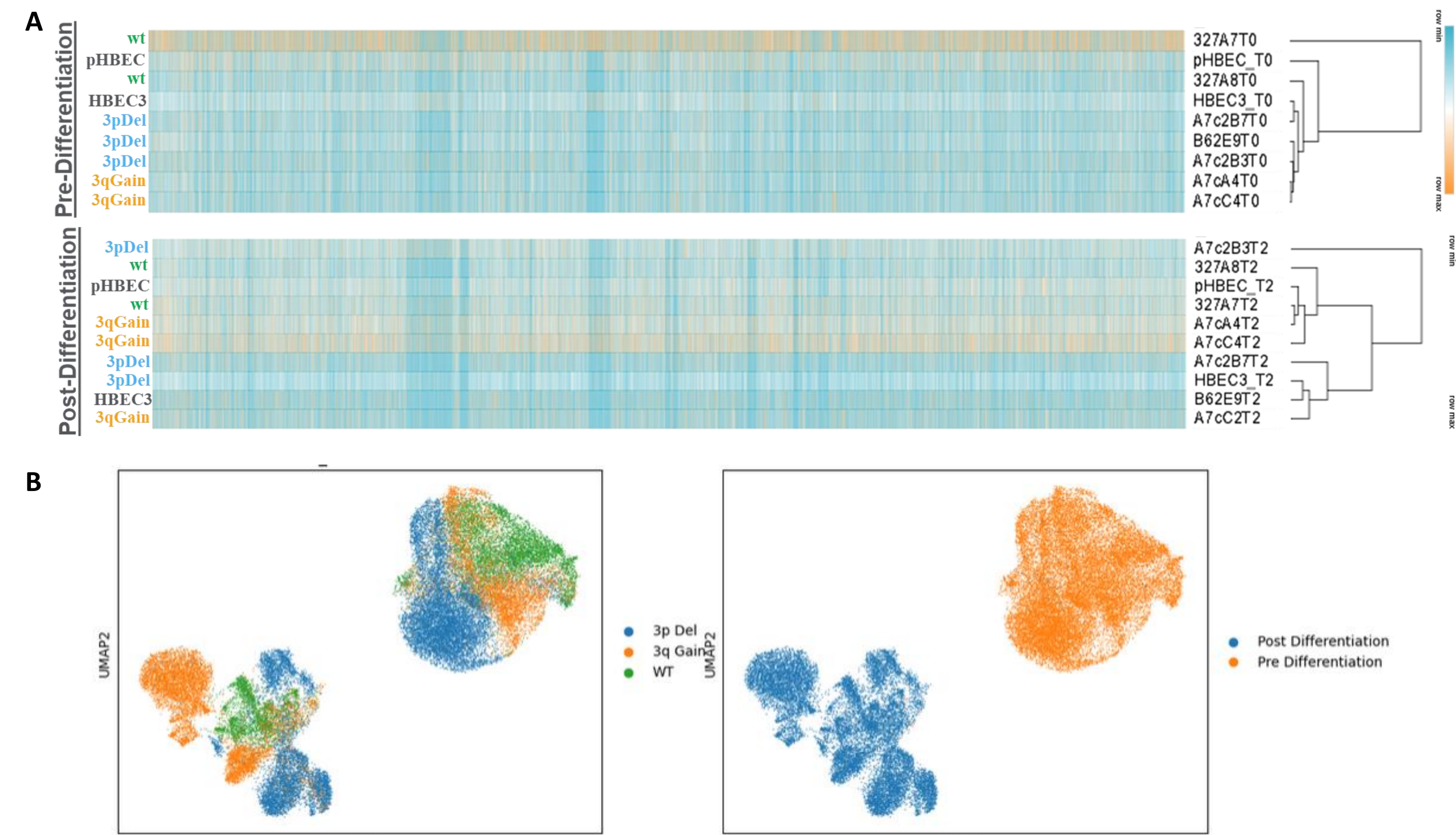


Fig 4: A) Bulk RNAseq hierarchical clustering for pHBEC, HBEC3 and AALE (WT, 3pDel and 3q Gain)-derived organoids before and after differentiation. **B)** scRNAseq clustering for AALE (WT, 3pDel and 3q Gain)-derived organoids before and after differentiation.

4. Specific cell-signatures correlate with 3pDel and 3qGain genotypes

Further analysis of our differentiated organoids scRNAseq data revealed that certain cell signatures were enriched in specific genotypes. Specifically, we identified clusters 4 and 10 (Fig. 5A) to correspond to secretory cell types by gene expression signature (Fig. 5B), which were almost entirely represented by 3pDel cells (90.3% and 88.2% respectively) (Fig. 5C). At the same time, clusters 2, 7 and 11 were classified as submucosal gland (SMG) basal cells based on their gene expression signatures (Fig. 5D) showing these cluster a clear enrichment in 3qGain cells (97.7%, 73% and 63.5% respectively) (Fig. 5E).

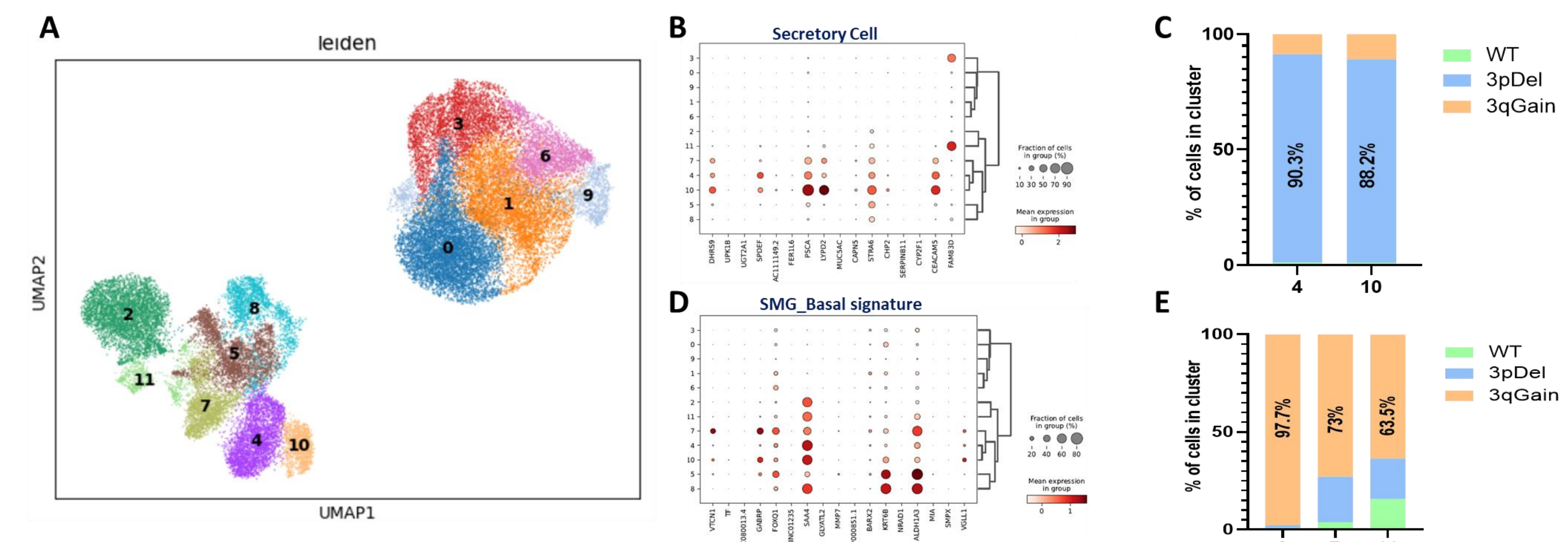


Fig 5: A) scRNAseq clustering from our AALE organoids (WT, 3pDel and 3qGain) before and after differentiation. **B)** Dot plot showing the expression and abundance of secretory cell markers across the identified clusters **C)** Percentage of each AALE genotype in secretory cell clusters. **D)** Dot plot showing the expression and abundance of SMG basal cell markers across the identified clusters **E)** Percentage of each AALE genotype in SMG clusters.

5. GSEA analysis identified increased NOTCH and TGFB signaling in 3pDel and 3qGain respectively

Using GSEA analysis we identified NOTCH signaling as an enriched pathway in 3pDel derived organoids (Fig. 6A). This is in line with previously described findings were sustained NOTCH signaling promoted secretory cell differentiation in lung basal cells, similarly to our scRNAseq observations. However, treatment with a NOTCH inhibitor (DAPT) did not rescue the phenotype during differentiation, indicating that other pathways are probably contributing to the observed 3pDel phenotype. In the case of 3qGain organoids we detected an increased TGFB signaling (Fig. 6B). Interestingly, the use of a TGFB inhibitor (A-83-01) during differentiation showed increased clearance in the inner part of 3qGain organoids, suggesting a remodeling of the organoid architecture mediated by TGFB inhibition. Using gene expression data (Fig. 6C) and immunofluorescence (Fig. 6D) we observed and increased apoptosis in the inner part of the organoids paired with an upregulation of apoptosis related genes that was specific to 3qGain organoids and not observed in WT controls.

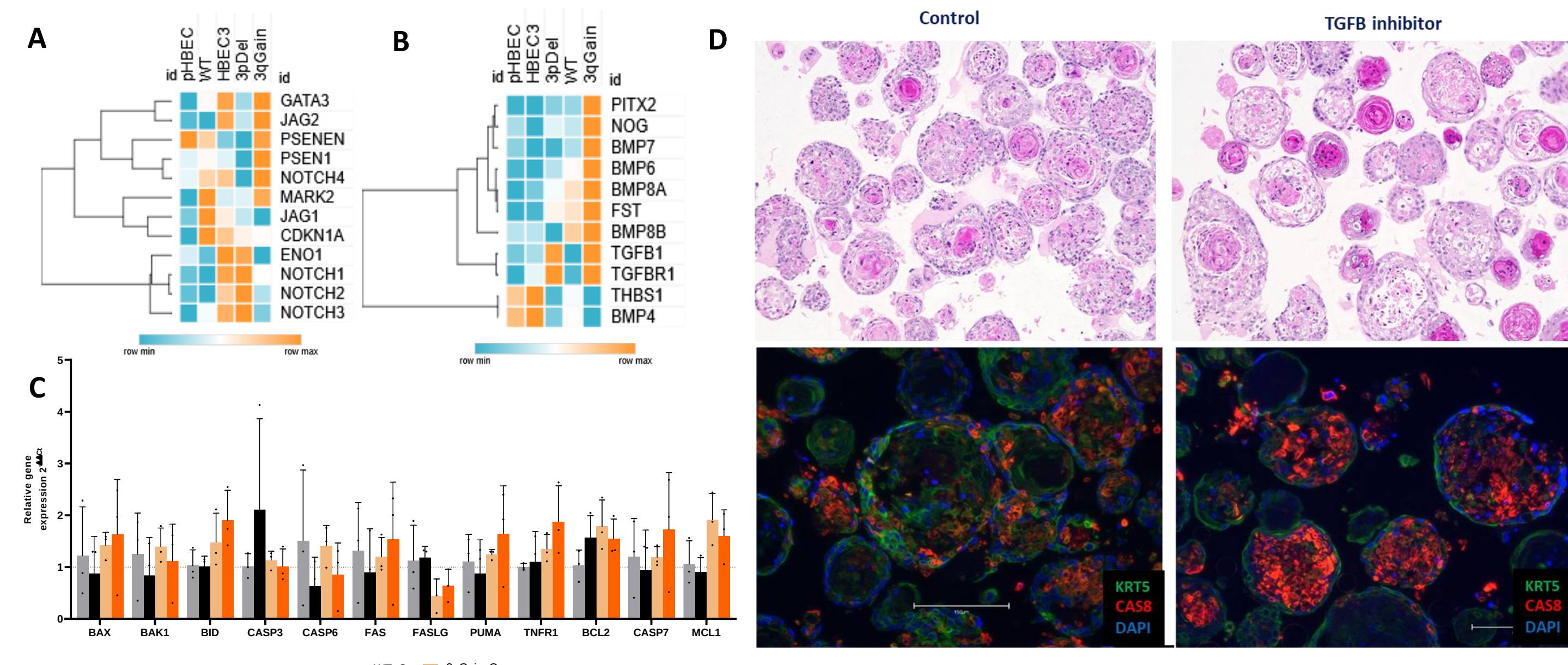


Fig 6: Gene expression FC of A) NOTCH and **B)** TGFB signaling genes in differentiated organoids. **C)** Apoptosis-related gene expression FC after TGFB inhibition in WT (gray:control, black:TGFB inh) and 3qGain (light orange:control; dark orange: TGFB inh) **D)** Representative H&E staining and immunofluorescence of 3qGain control and TGFB inh organoids after differentiation.

❖ Conclusions and Future directions

Our results show aneuploidy genotype-specific features upon the differentiation of basal-like cells in 3D. Furthermore, these results suggest that aneuploidy can impair the differentiation of cells toward specific cell types with abnormal phenotypes. Further research will determine the potential of targeting these alterations to promote terminal differentiation of aneuploid lung basal cells.