ORIGINAL ARTICLE

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A functional genomics approach to investigate the differentiation of iPSCs into lung epithelium at air-liquid interface

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Abstract

The availability of robust protocols to differentiate induced pluripotent stem cells (iPSCs) into many human cell lineages has transformed research into the origins of human disease. The efficacy of differentiating iPSCs into specific cellular models is influenced by many factors including both intrinsic and extrinsic features. Among the most challenging models is the generation of human bronchial epithelium at air-liquid interface (HBE-ALI), which is the gold standard for many studies of respiratory diseases including cystic fibrosis. Here, we perform open chromatin mapping by ATACseq and transcriptomics by RNA-seq in parallel, to define the functional genomics of key stages of the iPSC to HBE-ALI differentiation. Within open chromatin peaks, the overrepresented motifs include the architectural protein CTCF at all stages, while motifs for the FOXA pioneer and GATA factor families are seen more often at early stages, and those regulating key airway epithelial functions, such as EHF, are limited to later stages. The RNA-seq data illustrate dynamic pathways during the iPSC to HBE-ALI differentiation, and also the marked functional divergence of different iPSC lines at the ALI stages of differentiation. Moreover, a comparison of iPSC-derived and lung donor-derived HBE-ALI cultures reveals substantial differences between these models.

KEYWORDS

functional genomics, iPSC to HBE-ALI, open chromatin, transcriptional network, transcriptome

1 | INTRODUCTION

The technological advances that initiated the field of induced pluripotent stem cell (iPSC) biology 1,2 provided a new toolbox for

examining the establishment of cell identity and the organization of tissues. The availability of stem cells, which upon activation with defined stimuli can produce one of many different human tissue types, has facilitated many aspects of medical research (reviewed

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in 3). Here, we focus on the epithelium of the human airway that is intimately involved in the aetiology of many common respiratory diseases including chronic obstructive pulmonary disease (COPD), asthma and cystic fibrosis. Among the best resources for investigating human bronchial (HBE) and tracheal (HTE) epithelial cells are primary cultures derived from lung tissue and grown on plastic or differentiated on permeable supports.⁴⁻⁶ However, these are always in limited supply as they are dependent on the availability of suitable donor organs and tissue. More recently, robust protocols were developed to differentiate iPSCs into lung epithelial cells at air-liguid interface (ALI), thus modelling the HBE cells on permeable supports.⁷⁻¹⁶ Extensive characterization of the iPSC-to-ALI cell cultures has largely focused on their relevance to disease states (particularly cystic fibrosis ^{10,17}) and also generated elegant data on aspects of stem cell biology and the detailed lineages of the cell types.¹⁸⁻²⁴ However, complete maturation of iPSC-derived ALI cultures into functional HBE-ALI cells is often challenging. This obstacle may depend not only on the differentiation capacity of individual iPSC lines but also on the absence of appropriate biochemical and/or physical cues.²⁵⁻²⁷ Our goal was to develop a robust and unbiased functional genomics pipeline to benchmark the differentiation of iPSC lines from different sources cultured according to diverse protocols in individual laboratories. Here, we utilize genome-wide methods to examine the gene regulatory networks that drive the pathways of differentiation from iPSCs to ALI cultures and compare the model ALI cultures with HBE-ALI cells. By intersecting open chromatin data (generated by Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq)²⁸) with the transcriptome (generated by RNA-seq) of defined stages of differentiation of the iPSC to ALI pathway, we reveal the dynamic changes in the transcriptional networks that drive these alterations. We also show the divergence in the molecular signatures of iPSC-derived ALI and lung donor-derived HBE-ALI cells, which may provide insights into the factors that are required to overcome the hurdles of complete maturation of the model ALI cells into mature HBE cells.

2 | MATERIALS AND METHODS

2.1 | Cell culture

iPSC lines CR0000008 (Cell line ID: ND2.0), CR0000011 (Cell line ID: ND1.4) were purchased from RUCDR Infinite Biologics https://www.nimhgenetics.org/stem_cells/crm_lines.php (www. rucdr.org). CWRU205 was donated by Dr Paul J. Tesar (Dept. of Genetics and Genome Sciences, Case Western Reserve University). All three lines were from male donors. Differentiation of iPSC to ALI culture was based on the method by Wong et al,¹⁰ with minor modifications in coating of the membrane inserts (Corning 346012 mm Transwell inserts) for the differentiation protocol. After testing different coating methods described by others,^{10,11} we found Matrigel (Corning 35623, 1/60 dilution) with collagen IV (60 μg/mL) supported the most robust and consistent

ALI cultures. The stages of differentiation were confirmed by 1) RT-qPCR assays specific for NKX2.1, FOXA2, SOX2, SOX9, SOX17, MUC5AC, MUC16, FOXJ1, TP63 and KRT5 transcripts, using primers shown in Table S1; and specific antibodies to CXCR4 (CD184, BD Pharmigen 5559740), SOX17 (BD Pharmigen 562594), NKX2.1 (TTF1; Abcam ab76013), SOX2 (GeneTex GTX101507), FOXA2 (Abcam ab60721) for FACS at DE and AFE stages; and immunofluorescence for NKX2.1, FOXA2, SCGB1A1 (CC10, Santa Cruz Biotechnology (SCB) sc-365992) and MUC5B (SCB sc-21768) at AFE, LP3a and ALIw5 stages.

Donor-derived HBE cells were obtained from the Marsico Lung Institute CF Center Tissue Procurement and Cell Culture Core (University of North Carolina (UNC), Chapel Hill, NC) and cultured according to the published protocols^{5,6} in accordance with relevant guidelines. The cells were obtained under protocol #03-1396 approved by the University of North Carolina at Chapel Hill Biomedical Institutional Review Board. All donors or their authorized representatives provided informed consent for research use of explanted lungs. This work was also approved by the Case Western Reserve University Institutional Review Board.

2.2 | RNA preparation, reverse transcription quantitative PCR (RT-qPCR) and RNA-seq

Total RNA was extracted from iPSCs at different stages of differentiation using TRIzol (Invitrogen) using their protocol. RT-qPCR was done using our standard protocols²⁹ with primers shown in Table S1. RNA-seq (SR 50bp) was performed as described previously.³⁰

2.3 | ATAC-seq

ATAC-seq was performed on 50 000 cells as previously described²⁸ with the following minor modifications. The initial cell pellet was washed 1-2 times in 100 µL cold PBS and centrifuged for 5 minutes at 500 \times g at 4°C. The washed cell pellet was resuspended in 100 µL cold lysis buffer (100 mmol/L Tris-Cl, pH 7.4; 10 mmol/L NaCl; 3mmol/L MgCl₂; 0.1% (v/v) IGEPAL CA-630, freshly prepared), incubated on ice for 10 minutes and centrifuged for 10 minutes at $500 \times g$ at 4°C. The nuclear pellet was washed once in 50 μ L homemade TD buffer³¹ (20 mmol/L Tris(hydroxymethyl)aminomethane; 10 mmol/L MgCl₂; 20% (v/v) dimethylformamide) and centrifuged for 5 minutes at $500 \times g$ at 4°C, before proceeding with tagmentation using Nextera Tn5 Transposase and commercial 2X TD buffer (Illumina). Following preparation, the final ATAC-seq libraries were purified with Agencourt AMPure XP magnetic beads (Beckman-Coulter) at a sample to bead ratio of 1:1.2, following the manufacturer's protocol, and eluted into 20 µL Buffer EB (Qiagen). ATAC-seq library size distributions were visualized by TapeStation (Agilent) and quantified using the KAPA Library Quantification Kit (Roche). Six to seven libraries were pooled, per lane, and sequenced on a HiSeq4000 (Illumina) using 100 bp paired-end sequencing.

RNA-seg analysis 2.4

Raw reads were aligned with STAR 2.6 (https://github.com/alexd obin/STAR).³² Aligned reads were then assigned to genomic features with featureCounts version 1.6.3 in the Subread package (http:// subread.sourceforge.net/)³³ and differential gene expression was analysed using DEseq2 version 1.22.1 (https://www.bioconduct or.org/packages/release/bioc/html/DESeq2.html).³⁴

Raw reads of six untreated healthy donor-derived HBE-ALI samples were acquired from the GEO database Series GSE97036.³⁵ Data sets were processed using the same pipeline. The cells used in this series were as described above for donor-derived HBE cells.

2.5 Gene ontology analysis

Differentially expressed genes were filtered to enrich for those with a fold change ≥1.5 and Benjamini-Hochberg adjusted P-value ≤ 0.01. Gene lists were read into the PANTHER gene ontology database (http://pantherdb.org/)³⁶ using the biological processes term. Results were further validated using gProfiler. Statistically significant results were filtered for categories passing a P-value of 0.001 with the Bonferroni correction for multiple testing.

2.6 ATAC-seq data processing

Raw reads from the HiSeq4000 were processed using the ENCODE ATAC-seq pipeline (Dec 2018)(https://github.com/kundajelab/ atac-seq-pipeline). Replicates were used to generate peaks with an IDR threshold of 0.5. Analysis of enriched motifs within the IDR peaks was performed with HOMER (4.7.2g) (http://homer.ucsd. edu/homer/index.html).³⁷ PCA and heatmaps were generated by DiffBind (2.10.0) (http://bioconductor.org/packages/release/bioc/ vignettes/DiffBind/ inst/doc/DiffBind.pdf).38





2.7 | Binding and expression target analysis BETA

Differential gene expression for STAR aligned reads was analysed using the Cufflinks suite v2.2.1. (http://cole-trapnell-lab.github.io/cufflinks/).³⁹ Cuffdiff output for pairwise comparisons was processed against ATAC-seq peak files for each stage in the pair independently using the BETA software version 1.0.7 (http://cistrome.org/BETA/)⁴⁰ to determine peak distribution at differentially expressed genes (DEGs). Peaks were chosen within 20 kb from the gene body, and function prediction was determined by the rank product of the estimated regulatory potential of factor binding and change in expression of the gene target.

2.8 | Immunofluorescence

NHEY

iPSC ALI cultures (week 5) on membrane filters were harvested and fixed in 4% paraformaldehyde (in PBS), embedded in paraffin and cut into 5-µm sections. After deparaffinization and rehydration, antigen retrieval was performed by heating with citric acid (10 mmol/L, pH 6.0) in a 98°C water bath for 45 minutes. The tissue sections were subsequently washed 3 times in blocking solution (1% BSA and 0.05% Saponin in PBS). The sections were incubated with the primary antibodies (diluted in PBS containing 0.1% BSA) overnight at 4°C. The sections were then washed 3 times in PBS containing 0.05% Tween 20, followed by incubation with secondary antibodies (diluted in PBS with 0.1% BSA) for 1 hour at room temperature. After 3 washes and counterstaining with 4,6-diamidino-2-phenylindole (DAPI), the samples were mounted with ProLong Gold Antifade Mountant (Invitrogen). The specimens were imaged on a fluorescence microscope (Leica DM 6000). Primary antibodies used were as follows: NKX2.1 (TTF1; Abcam ab76013), FOXA2 (Abcam ab60721) and CC10 (SCB sc-365992). Secondary antibodies used were as follows: Rhodamine Red[™]-X (RRX) AffiniPure Donkey Anti-Mouse IgG (H + L) (Jackson ImmunoResearch 715-295-151) and Alexa Fluor 488 (AF488) AffiniPure Donkey Anti-Rabbit IgG (H + L) (Jackson ImmunoResearch 711-545-152).

3 | RESULTS

Our experimental and analysis pipelines are summarized in Figure 1. In initial experiments, we differentiated three iPSC lines, ND2.0, ND1.4 and CWRU205, which came from 2 different sources. We followed the standard protocols as described in the methods section to culture the iPSCs and differentiate them into airway epithelial cells at ALI (Figure 1A). Cells were collected for ATAC-seq and RNA-seq at 6 time-points: definitive endoderm (DE), anterior foregut endoderm (AFE) and lung progenitor cells 3a, 3b (LP3a, LP3b), at air-liquid interface after weeks 3 and weeks 5 (ALIw3 and ALIw5). The ND1.4 iPSC line repeatedly failed to differentiate fully to the ALI stage (either through not adhering to membrane supports at the transition to AFE cells, or due to reduced cell proliferation starting at LP3a), so this line was not analysed further. Open chromatin and RNA-seq data were derived from cells in multiple differentiation processes of the ND2.0 and CWRU205 lines. However, data presented here are from one differentiation process where these 2 lines were grown in parallel using the same batches of culture media, supplements and culture inserts, to minimize variation that was not inherent to the cells. The RNA-seq and ATAC-seq pipeline are summarized in Figure 1B.

3.1 | Open chromatin mapping through differentiation from iPSC-derived definitive endoderm through to air-liquid interface lung epithelial cell cultures

We first used ATAC-seq to identify the regions of open/active chromatin at each developmental stage and combined data from the ND2.0 and CWRU205 lines to generate a reproducible peak set by irreproducible discovery rate (IDR) analysis. Principal component analysis (PCA) (Figure 2A) showed close concordance between the open chromatin profiles of the two donors at each time-point from DE to LP3b. In contrast, at the ALIw3 and ALIw5 stages the within donor similarities were greater than time-pointassociated features. Of note, the open chromatin profiles of the DE stage were most divergent from all other stages of differentiation. We also examined the IDR peak distributions across the different genomic regions (intergenic, intronic, promoter [within 2 kb 5' of the transcriptional start site] or other) and found these to be largely consistent between differentiation stages (Figure 2B). Next, we performed transcription factor (TF) motif enrichment analysis on the IDR peaks at each stage of differentiation using HOMER (Figure 2C, Figure S1). Focusing on known motifs, many factors are significantly enriched in peaks of open chromatin including binding sites for architectural proteins CCCTC-binding factor (CTCF)⁴¹ and Brother of the Regulator of Imprinted Sites (BORIS),⁴² also known as CCCTC-binding factor-like (CTCFL). CTCF is the most overrepresented motif at DE, AFE and ALIw3 stages (Figure 2C i, ii, iv). Consistent with its pivotal role in organizing higher order chromatin structure genome-wide, the CTCF motif is among the top 7 motifs at all stages of differentiation (Figure 2C, Figure S1). Among TFs with pioneer activity (opening chromatin to increase accessibility for other TFs) and also with

FIGURE 2 Analysis of open chromatin through iPSC to ALI differentiation. A, Principal component analysis (PCA) plot showing relationships between open chromatin at different stages of differentiation from the ND2.0 (ND) and CWRU205 (CW) iPSC lines. Each developmental stage is colour coded and numbered according to the inset panel. B, Genomic distribution of open chromatin peaks at each stage of differentiation from an IDR analysis of the ND2.0 and CWRU205 iPSC lines. Peaks called as: intergenic, intronic, promoter (defined as 2 kb upstream of the transcription start site) or other. C, HOMER analysis showing overrepresented binding motifs for known transcription factors under open chromatin peaks at 4 developmental stages i) DE, ii) AFE, iii) LP3b, iv) ALIw3



1 02002 00000000000000000000000000000000	Rank		Name	P-value	Target Sequences with Motif	Background Sequences with Motif
2 SEESSAGCCCCCCCCTSAGTCSC CTCFL (BORIS) 14-681 3.69% 1.45% 3 AGATAASC GATA3 16-681 6.21% 3.16% 4 SEAGATAASC GATA4 16-683 4.29% 1.90% 5 SECTTATCTS GATA2 16-633 4.29% 1.90% 6 SAGATAASC GATA1 16-633 4.29% 3.87% 1.71% 7 SEGATTAS GSC 16-174 11.2% 8.89% 9 SATAATCC CRX 16-148 3.73% 5.55% 10 SCATTETS SOX10 16-140 9.81% 7.84% 12 SCATTETS SOX10 16-117 7.72% 5.57% 13 SCOATTETS SOX6 16-109 6.85% 5.14% 14 SESTTETSS SOX4 16-90 3.82% 2.84% 14 SESTTETSAS SOX4 16-90 3.82% 2.84% 14 SESTETTACTS SOX4 16-90 3.85%	1	ATASIGCCAFCT>GGF&A	CTCF	1e-1207	2.70%	0.55%
3 AGATAASS GATA3 1e-664 8.52% 4.86% 4 SERCATAASS GATA4 1e-63 6.21% 3.16% 5 SEGTATACES GATA2 1e-33 4.29% 1.90% 6 SACATTAACSS GATA1 1e-62 3.97% 1.71% 7 SSSATTAS GSC 1e-174 11.22% 8.80% 9 SETAATCCC CRX 1e-140 9.81% 7.3% 5.95% 10 OCATTGTA EOMES 1e-140 9.81% 7.44% 12 SCATTGTASS SOX3 1e-140 9.81% 7.44% 13 SCATTGTASS SOX2 1e-140 9.81% 7.44% 14 SEQTTGTASS SOX6 1e-140 9.81% 7.44% 14 SEQTTGTASS SOX6 1e-140 9.81% 7.44% 15 TAATGCS PITX1 1e-80 2.01% 7.86% 16 AGAGTASS PITX1 1e-93 3.85% 2.46% 17 SCTTGTACS SOX4 1e-94 4.28%	2	STSTREFCCCCCCCTSSIGGS	CTCFL (BORIS)	1e-681	3.69%	1.45%
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14 SQATTOTEXE SOX6 1e-109 6.58% 5.14% 15 TAATGCRS PITX1 1e-88 20.10% 17.80% 16 AAGTTAAACA FOXA1 1e-90 3.82% 2.46% 17 SCITTGTICS SOX4 1e-90 3.82% 2.84% 18 ASCTGTSAAQQ TBX5 1e-73 3.95% 3.03% 20 ACGTCTSAAQ TBX5 1e-64 4.28% 3.88% 21 SCCCCCCCCCCCS SP1 1e-64 4.88% 5.55% 23 SETGTTTACSE FOXA2 1e-60 2.88% 2.18% 24 ASCCAATSES NFY 1e-60 4.06% 3.13% 24 ASCCAATSES FOSL1 1e-87 4.06% 3.13% 2 XAGTGAATCAXS FOSL1 1e-87 2.12% Macgroun 3 20TGAATCAXS FOSL1 1e-87 2.12% 3.13% 2 XAGTGAATSES NFY 1e-10 4.65% <t< th=""><th>13</th><th>SCCATTOTIS</th><th>SOX2</th><th>1e-111</th><th>3.94%</th><th>2.84%</th></t<>	13	SCCATTOTIS	SOX2	1e-111	3.94%	2.84%
15 TAATECES PITX1 1e-88 20.10% 17.80% 16 AAAGTAAAÇA FOXA1 1e-92 3.40% 2.46% 17 SCITTGTES SOX4 1e-90 3.82% 2.84% 18 ASGTGTEAAAQ TBX21 1e-78 4.22% 3.25% 19 CQCACGAGGSSS Unk. ESC element 1e-70 16.83% 3.03% 20 AGGTGTEAA FBX5 1e-60 4.88% 3.38% 21 SCCCCCCCCCCCS SP1 1e-64 4.28% 3.38% 22 242TAATTAS LHX3 1e-60 2.88% 2.18% 23 SZTSTTTACCATA FOXA2 1e-60 2.88% 2.18% 24 ASCCAATEXS NFY 1e-61 4.65% 3.13% 24 ASCCAATEXS FOSL1 1e-77 1.48% 0.81% 2 X26CTGASTCATS FOSL2 1e-77 1.48% 0.81% 2 X26TGASTCATS JUN 1e-51 2.03% <th>14</th> <th>SSATTGT SSE</th> <th>SOX6</th> <th>1e-109</th> <th>6.58%</th> <th>5.14%</th>	14	SSATTGT SSE	SOX6	1e-109	6.58%	5.14%
16 AAQTAAAQA FOXA1 1e-32 3.40% 2.46% 17 \$CITTGTIQS SOX4 1e-30 3.82% 2.84% 18 A>GTBAAAQ TBX21 1e-78 4.22% 3.25% 19 C&CACCAGGSSS Unk. ESC element 1e-73 3.95% 3.03% 20 AGGTGJSA TBX5 1e-70 16.83% 15.03% 21 \$CCCCCCCCCCCS SP1 1e-64 4.28% 3.38% 22 &XETGTTTACASA LHX3 1e-64 4.28% 3.38% 23 \$ZETGTTTACASA FOXA2 1e-60 2.88% 2.18% 24 ASCCAATSXS NFY 1e-61 4.66% 3.13% 24 ASCCAATSXS FOSL1 1e-77 1.48% 0.81% 2 XEGTGASTCAIXS FOSL1 1e-71 2.30% 1.46% 3 #GTGASTCAIXS JUN 1e-67 1.10% 0.57% 3 #GTGASTCAIXS JUN 1e-67 1.40%<	15	TAATCCS	PITX1	1e-98	20.10%	17.80%
17 SCITTGILQS SOX4 1e-90 3.82% 2.84% 18 ASCTGIRAGE TBX21 1e-78 4.22% 3.25% 19 C&CACCACGESS Unk. ESC element 1e-73 3.95% 3.03% 20 AGETGIRA TBX5 1e-70 16.33% 15.03% 21 SCCCCCCCCCCS SP1 1e-64 4.28% 3.38% 22 SASTATTAS LHX3 1e-60 2.88% 2.18% 23 SATCATTAS NFY 1e-60 4.06% 3.21% 24 ASCCAATERS NFY 1e-60 4.06% 3.3% 2 ZSATGATCAIRS NFY 1e-10 4.65% 3.13% 2 ZSATGAATERS FOSL1 1e-77 1.48% 0.81% 3 SATGASTCAIRS FOSL2 1e-77 1.48% 0.81% 4 ATGASTCAIRS ATF3 1e-70 2.33% 1.49% 5 SATGASTCAIRS JUN 1e-50 5.91% <	16		FOXA1	1e-92	3.40%	2.46%
AGGTOTICAGE TBX21 1e-78 4.22% 3.25% 19 C2CAGCAGGS26 Unk. ESC element 1e-73 3.95% 3.03% 20 AGGTOTICAGA TBX5 1e-70 16.83% 15.03% 21 SECCCCCCCCCCC SP1 1e-64 4.28% 3.38% 22 AGGTOTICAGE SP1 1e-64 4.28% 3.38% 22 AGGTOTICAGE SP1 1e-64 4.28% 3.38% 23 SATISTTACES FOXA2 1e-60 2.88% 2.18% 24 AGCCAATESS NFY 1e-60 4.06% 3.13% 24 AGCCAATESS NFY 1e-10 4.65% 3.13% 2 X20TGAETCAES FOSL1 1e-87 2.12% 125% 3 SOTGAETCAES FOSL2 1e-77 1.48% 0.81% 4 X20GAETCAES FOSL2 1e-77 1.48% 0.81% 3 SOTGAETCAES FOSL2 1e-77 1.48% 0.87%<	17	SCITTCITCS	SOX4	1e-90	3.82%	2.84%
Image: Construction of the second s	18	ASGTGTSAAA	TBX21	1e-78	4.22%	3.25%
Description Description <thdescription< th=""> <thdescription< th=""></thdescription<></thdescription<>	19	CACAGCAGGGGG	Unk. ESC element	1e-73	3.95%	3.03%
21 2CCCCCCCCCCS SP1 1e-64 4.28% 3.38% 22 222TAATTAS LHX3 1e-64 6.68% 5.55% 23 SATGTTTACATAS LHX3 1e-60 2.88% 2.18% 24 ASCCAATSKS NFY 1e-60 4.06% 3.21% iii LP3b Known Motifs Name P-value % of Target Backgroup % of thout 1 ASCCAATSKS NFY 1e-10 4.65% 3.13% 2 X26TGAETCATS FOSL1 1e-87 2.12% 1.25% 3 64TGAETCATS FOSL2 1e-71 1.48% 0.81% 4 78TGAETCATS FOSL2 1e-71 2.33% 1.46% 5 SATGAETCATS JUN 1e-67 1.10% 0.57% 6 SATGAETCATS JUN 1e-67 1.03% 0.6% 8 SCCCCCCCCCCS SP1 1e-30 2.33% 1.48% 9 MCAETCATS JUN 1e-50 5.91	20	AGGTCIÇA	TBX5	1e-70	16.83%	15.03%
22 23 STIATTA LHX3 1e-64 6.68% 5.55% 23 SETETTIACEE FOXA2 1e-60 2.88% 2.18% 24 ASCCAATESS NFY 1e-60 4.06% 3.21% iii LP3b Known Motifs Name P-value % of Target Sequences with Motif Sequences with Motif 1 ASCCAATESS NFY 1e-10 4.65% 3.13% 2 Z20TGASTCAES FOSL1 1e-87 2.12% 1.25% 3 SOTGASTCAESS FOSL2 1.677 1.48% 0.81% 4 X4TGASTCAESS FOSL1 1e-70 2.33% 1.46% 5 SOTGASTCAESS JUN 1e-67 1.10% 0.57% 6 SOTCCCCCCCCCS SP1 1e-50 2.33% 1.47% 10 SEGGATYF TEAD 1e-31 2.33% 1.77% 10 SEGCACCCCCCCCS SP1 1e-51 5.91% 4.64% 9 ACGASTCAESS JUN <td< th=""><th>21</th><th>SCCCCCCCCCS</th><th>SP1</th><th>1e-64</th><th>4.28%</th><th>3.38%</th></td<>	21	SCCCCCCCCCS	SP1	1e-64	4.28%	3.38%
Laboration Lines FOXA2 1e-60 2.88% 2.18% 23 SETITITACEZEA FOXA2 1e-60 2.88% 2.18% 24 ASCCAATEXS NFY 1e-60 4.06% 3.21% iii LP3b Known Motifs Rank Motif Name P-value % of Sequences Sequences with Motif % of arget sequences with Motif % of arget sequences sequences % of arget sequences with Motif % of arget sequences sequences 1 ASCCAATEXS NFY 1e-10 4.65% 3.13% 2 XSATCAATEXS FOSL1 1e-87 2.12% 0.81% 3 SQTGASTCAIXS BATF 1e-70 2.33% 1.46% 4 XATGASTCAIXS ATF3 1e-50 2.53% 0.67% 6 SQTGASTCAIXS CTCF 1e-62 1.23% 0.67% 8 SQCCCCCCCCCCS SP1 1e-50 2.53% 1.77% 9 ATGASTCAIXS TEAD 1.61% 1.61% 1.77% 10 SQLGCCCCCCCCCS SP1 <	22	ΑΥΣΤΑΛΤΤΑΩ	LHX3	1e-64	6.68%	5.55%
Part of the construction NFY 1e-60 4.06% 3.21% III LP3b Known Motifs Name P-value % of Target Sequences % of Backgroun 1 ASCCAATEXS NFY 1e-10 4.65% 3.13% 2 X20TGAETCAEX FOSL1 1e-87 2.12% 1.25% 3 SOTGAETCAEX FOSL2 1e-77 1.48% 0.81% 4 XATGAETCAEX FOSL2 1e-77 1.48% 0.81% 5 XATGAETCAEX ATF3 1e-70 2.33% 1.49% 6 XATGAETCAEX ATF3 1e-70 2.33% 1.49% 6 XATGAETCAEX ATF3 1e-50 2.53% 1.77% 7 ASAESEGCCCCCCCC SP1 1e-56 5.91% 4.64% 9 ATGAETCAEX TEAD 1e-40 2.26% 1.61% 11 XXXXXXXX TEAD 1e-31 2.72% 1.60% 12 CCCACACCCA KLF4 1e-31	23	SETGTTTACAZA	FOXA2	1e-60	2.88%	2.18%
III LP3b Known Motifs Name P-value % of sequence: vith Motif % of Mackgroun % of sequence: vith Motif	24	AGCCAATSSG	NFY	1e-60	4.06%	3.21%
1 ASCCATEXS NFY 1e-10 4.65% 3.13% 2 Z&TGAETCAES FOSL1 1e-87 2.12% 1.25% 3 SOTGAETCAES FOSL1 1e-77 1.48% 0.81% 4 ZETGAETCAES FOSL2 1e-77 1.48% 0.81% 5 SATGAETCAES FOSL2 1e-70 2.33% 1.49% 6 SATGAETCAES ATF3 1e-70 2.33% 1.49% 6 SATGAETCAES JUN 1e-67 1.10% 0.57% 7 AEASECCCAPCTSSTGGSSA CTCF 1e-62 1.23% 0.67% 8 SCCCCCCCCCCS SP1 1e-50 2.53% 1.77% 9 AGAETCAES JUN 1e-51 2.44% 1.79% 11 STESSAESCCCCCCCCCS SP1 1e-37 2.44% 1.79% 12 GCCCACCCCA KLF4 1e-34 3.41% 2.66% 13 GOSSGGG MAZ 1e-31 2.72% 2.	 LP	SD KNOWN MOUNS			/0 01	/0 01
2 X&TCASTCATS FOSL1 1e-87 2.12% 1.25% 3 @STGASTCATS FOSL2 1e-77 1.48% 0.81% 4 X*TGASTCATS BATF 1e-70 2.30% 1.46% 5 %STGASTCATS BATF 1e-70 2.33% 1.49% 6 %STGASTCATS JUN 1e-67 1.0% 0.57% 7 #ASTGASTCATS JUN 1e-67 1.0% 0.57% 8 SCCCCCCCCCCCS SP1 1e-56 5.91% 4.64% 9 ATGASTCATS JUN 1e-67 2.33% 1.77% 10 STGGATTS TEAD 1e-40 2.26% 1.61% 11 \$STGGATTS TEAD 1e-32 2.44% 1.79% 12 SCCCACACCCA KLF4 1e-31 3.41% 2.66% 13 GOSSGGG MAZ 1e-31 0.31% 1.60% 14 SATGACTCACACA NFE2 1e-31 0.31% 1.43% <	Rank	k Motif	Name	P-value	Target Sequences with Motif	Background Sequences with Motif
3 3 3 40 CABYS FOSL2 1e-77 1.48% 0.81% 4 TATCABICALXS BATF 1e-71 2.30% 1.46% 5 SATGASTCALXS ATF3 1e-70 2.33% 1.49% 6 SATGASTCALXS ATF3 1e-70 2.33% 1.49% 6 SATGASTCALXS AUN 1e-67 1.10% 0.57% 6 SATGASTCALXS CTCF 1e-62 1.23% 0.67% 8 SCCCCCCCCCCCS SP1 1e-50 2.53% 1.77% 10 STAGGATYS TEAD 1e-40 2.26% 1.61% 11 STATCACCA KLF4 1e-31 2.44% 1.79% 12 GCCCACCCCA KLF4 1e-31 2.44% 1.79% 12 GCCCACCCCA KLF4 1e-31 0.31% 1.03% 13 GACACCAACCA NFE2 16-31 0.31% 1.03% 14 SATGACTCAGCA NFE2 <t< th=""><th>Rank</th><th></th><th>Name</th><th>P-value 1e-110</th><th>Target Sequences with Motif 4.65%</th><th>Background Sequences with Motif 3.13%</th></t<>	Rank		Name	P-value 1e-110	Target Sequences with Motif 4.65%	Background Sequences with Motif 3.13%
4 X&TGA&TCAI BATF 1e-71 2.30% 1.46% 5 X&TGA&TCAIXX ATF3 1e-70 2.33% 1.49% 6 X&TGA&TCAIXX ATF3 1e-70 2.33% 1.49% 6 X&TGA&TCAIXX ATF3 1e-70 2.33% 1.49% 6 X&TGA&TCAIXX ATF3 1e-70 2.33% 1.49% 7 & & & & & & & & & & & & & & & & & & &	Rank 1 2	Motif ASCCAATSSS SSATGASTCAES	Name NFY FOSL1	P-value 1e-110 1e-87	Target Sequences with Motif 4.65% 2.12%	Background Sequences with Motif 3.13% 1.25%
5 %ATGASTCAIX% ATF3 1e-70 2.33% 1.49% 6 %ATGASTCAIX% JUN 1e-67 1.10% 0.57% 7 AZASZGCCAGCT%STGGG% CTCF 1e-62 1.23% 0.67% 8 SCCCCCCCCCCS SP1 1e-50 2.53% 4.64% 9 ATGASTCAIX% JUN 1e-50 2.53% 4.64% 10 %STGGAATP% TEAD 1e-40 2.26% 1.61% 11 %STGSASSGCCCCCCCCT%STGGG CTCFL (BORIS) 1e-37 2.44% 1.79% 12 GCCAACCCA KLF4 1e-34 3.41% 2.66% 13 GCSSGGGG MAZ 1e-31 0.31% 0.13% 14 %ATGATCAACA FOXA1 1e-31 0.31% 0.13% 15 FAAGTAAACA FOXA1 1e-32 1.63% 1.43% 15 FAAGTAAACA FOXA1 1e-38 0.86% 0.54% 16 SCAGAATGL BACH2 1e-28 0.8	Rank 1 2 3	ASCCAATSSS SSCCAATSSS SSCCAATSSS SSTGASTCAES SSTGASTCAESS	Name NFY FOSL1 FOSL2	P-value 1e-110 1e-87 1e-77	Target Sequences with Motif 4.65% 2.12% 1.48%	Background Sequences with Motif 3.13% 1.25% 0.81%
6 STCATES JUN 1e-67 1.10% 0.57% 7 ACASTCATES JUN 1e-67 1.10% 0.57% 7 ACASTCATES CTCF 1e-62 1.23% 0.67% 8 SCCCCCCCCCCCS SP1 1e-56 5.91% 4.64% 9 ATCASTCATES JUN 1e-50 2.53% 1.77% 10 SEGCAATES JUN 1e-50 2.53% 1.61% 11 SEGCACCCCCCCTSETEGGE CTCFL (BORIS) 1e-37 2.44% 1.79% 12 GCCACACCCA KLF4 1e-34 3.41% 2.66% 13 GCESCGGG MAZ 1e-31 0.31% 0.13% 14 SATGACTCACCA NFE2 1e-31 0.31% 0.13% 15 FAAGTAACA FOXA1 1e-39 1.43% 16 SCCGCAATGE TEAD2 1e-29 1.95% 0.44% 18 STCCTCAGTCAT NFE2L2 1e-28 0.27% 0.11%	Rank 1 2 3 4	ASCCAATESS SECCAATESS SECCAATESS SETGAETCAIS SETGAETCAIS SETGAETCAI	Name NFY FOSL1 FOSL2 BATF	P-value 1e-110 1e-87 1e-77 1e-71	Target Sequences with Motif 4.65% 2.12% 1.48% 2.30%	Background Sequences with Motif 3.13% 1.25% 0.81% 1.46%
7 AEAGLECCAPCTASTOGERA CTCF 1e-62 1.23% 0.67% 8 SCCCCCCCCCS SP1 1e-56 5.91% 4.64% 9 ATGACTCAISE JUN 1e-50 2.53% 1.77% 10 SETGCAATSE JUN 1e-50 2.53% 1.61% 11 SETGCACCCCCCTSETGGE CTCFL (BORIS) 1e-37 2.44% 1.79% 12 GCCCACCCCA KLF4 1e-34 3.41% 2.66% 13 GCGSSGGG MAZ 1e-32 17.62% 16.00% 14 SATGACTCACCA NFE2 1e-31 0.31% 0.33% 15 PAAGTAAACA FOX11 1e-31 2.72% 2.08% 16 SCOGGAGATOE TEAD2 1e-29 1.95% 1.43% 17 TAGCTCACTCA BACH2 1e-28 0.86% 0.54% 18 PTCCTGASTCA BACH2 1e-28 0.87% 0.11% 19 PCCCECTGASC KLF5 1e-28 0.37%	Rank 1 2 3 4 5	Motif ASCCAATSX9 X20TQASTCAIX SOTQASTCAIX X0TGASTCAIX X0TGASTCAIX X0TGASTCAIX	Name NFY FOSL1 FOSL2 BATF ATF3	P-value 1e-110 1e-87 1e-77 1e-71 1e-70	Target Sequences with Motif 4.65% 2.12% 1.48% 2.30% 2.33%	Background Sequences with Motif 3.13% 1.25% 0.81% 1.46% 1.46%
8 SCCCCCCCCCCS SP1 1e-56 5.91% 4.64% 9 ATCASTCAIS JUN 1e-50 2.53% 1.77% 10 %XICGAATY TEAD 1e-40 2.26% 1.61% 11 %XICGAATY TEAD 1e-37 2.44% 1.79% 12 GCCACACCCA KLF4 1e-37 2.44% 1.79% 13 GCCSACGCG MAZ 1e-32 17.62% 16.00% 14 &ATGACTCACCA NFE2 1e-31 0.31% 0.13% 15 #AAGTAACA FOXA1 1e-32 1.75% 2.08% 16 \$CCCGCATCACCA NFE2 1e-32 1.95% 1.43% 17 IGCTGASTCA BACH2 1e-28 0.86% 0.54% 18 #TCCTCACTCAT NFE2L2 1e-28 0.27% 0.11% 19 #GCGCGCGACCC KLF5 1e-28 3.01% 0.29% 20 AGATAASS GATA3 1e-24 5.03% 4.26	Rank 1 2 3 4 5 6	Motif SECCAATSXS SECTOASTCA3S SETOASTCA3S XETOASTCA3 SETGASTCA3S SETGASTCA3SS	Name FOSL1 FOSL2 BATF ATF3 JUN	P-value 1e-110 1e-87 1e-77 1e-71 1e-70 1e-67	Target Sequences with Motif 4.65% 2.12% 1.48% 2.30% 2.33% 1.10%	Background Sequences with Motif 3.13% 1.25% 0.81% 1.46% 1.49% 0.57%
9 ATCASTCAIS JUN 1e-50 2.53% 1.77% 10 \$\$\$IGAATYS TEAD 1e-40 2.26% 1.61% 11 \$\$\$\$IGGAATYS TEAD 1e-31 2.26% 1.61% 11 \$\$\$\$\$\$\$\$\$\$\$\$\$\$\$\$CCCCCCCCCCCCCCCCCCCCC	Rank 1 2 3 4 5 6 7	Motif SSCCAATSXS SSCTGASTCAEX SattGASTCAEX	Name NFY FOSL1 FOSL2 BATF ATF3 JUN CTCF	P-value 1e-110 1e-87 1e-77 1e-71 1e-70 1e-67 1e-62	Target Sequences with Motif 4.65% 2.12% 1.48% 2.30% 2.33% 1.10% 1.23%	Background Sequences with Motif 3.13% 1.25% 0.81% 1.46% 1.49% 0.57% 0.67%
10 SELECANTYS TEAD 1e-40 2.28% 1.61% 11 STREESEGCCCCCCTSELGOS CTCFL (BORIS) 1e-37 2.44% 1.79% 12 GCCACACCCA KLF4 1e-32 2.44% 1.79% 13 GCCSACCCACCCA KLF4 1e-32 2.44% 1.79% 14 SATGACTCACCA NFE2 1e-31 0.31% 1.03% 14 SATGACTCACCA NFE2 1e-31 0.31% 1.03% 15 AAACTAAACA FOXA1 1e-31 2.72% 2.08% 16 SCCACACTCACCA BACH2 1e-28 0.86% 0.54% 18 ATCCTCAGTCAI NFE2.2 1e-28 0.27% 0.11% 19 AGGACAGTGACC KLF5 1e-28 0.27% 0.11% 19 AGGACAGTGACC KLF5 1e-28 0.27% 0.11% 20 AGATAASS GATA3 1e-24 5.03% 4.26% 21 AGATAASS GATACCCCANTCCCC NRF	Rank 1 2 3 4 5 6 7 8	Motif ASCCAATSXS XSATGASTCA3XS XATGASTCA3XS XATGASTCA3XS XATGASTCA3XS XATGASTCA3XS XATGASTCA3XS SATGASTCA3XS SATGASTCA3XS SATGASTCA3XS SATGASTCA3XS SATGASTCA3XS SATGASTCA3XS SATGASTCA3XS	Name NFY FOSL1 FOSL2 BATF ATF3 JUN CTCF SP1	P-value 1e-110 1e-87 1e-77 1e-71 1e-70 1e-67 1e-62 1e-56	Target Sequences with Motif 4.65% 2.12% 1.48% 2.30% 2.33% 1.10% 1.23% 5.91%	Background Sequences with Motif 3.13% 1.25% 0.81% 1.46% 1.49% 0.57% 0.67% 4.64%
11 SXSSSGCCCCCCCTSGTGGC CTCFL (BORIS) 1e-37 2.44% 1.79% 12 CCCACACCCA KLF4 1e-34 3.41% 2.66% 13 GGSSGGGG MAZ 1e-32 17.62% 16.00% 14 SATGACTCACCA NFE2 1e-31 0.31% 0.13% 15 AAGTAAACA FOXA1 1e-31 2.72% 2.08% 16 SCAGGAATGU TEAD2 1e-29 1.95% 1.43% 17 ISGCTGAETCA BACH2 1e-28 0.86% 0.54% 18 STGCTGAETCA BACH2 1e-28 0.27% 0.11% 19 AGGCECIGGC KLF5 1e-28 14.67% 13.29% 20 AGATAASS GATA3 1e-24 5.03% 4.26% 21 AGCCGCGAACT ELF1 1e-24 5.03% 4.26% 23 SCCCGCAATGCGC NRF 1e-23 2.47% 1.95% 23 SCGCGGAATGG GATA4 1e-21 2.5	Rank 1 2 3 4 5 6 7 8 9	Motif ASCCAATSXS XXATGA&TCA3X ASTGA&TCA3X XATGA&TCA3 XATGA&TCA3X XATGA&TCA3XX XATGA&TCA3XX XATGA&TCA3XX ASCCCCCCCCCX ATGA&TCA3XX ASCCCCCCCCCX ATGA&TCA3XX	NAME FOSL1 FOSL2 BATF ATF3 JUN CTCF SP1 JUN	P-value 1e-110 1e-87 1e-77 1e-71 1e-67 1e-62 1e-56 1e-50	Target Sequences with Motif 4.65% 2.12% 1.48% 2.30% 2.33% 1.10% 1.23% 5.91% 2.53%	Background Sequences with Motif 3.13% 1.25% 0.81% 1.46% 1.46% 0.57% 0.67% 4.64% 1.77%
12 GCCACACCCA KLF4 1e-34 3.41% 2.66% 13 GCSSCGGC MAZ 1e-32 17.62% 16.00% 14 SATGACTCACCA NFE2 1e-31 0.31% 0.13% 15 FAAGTAACCA FOXA1 1e-31 2.72% 2.08% 16 SCAGCAATQL TEAD2 1e-29 1.95% 1.43% 17 TAGCTCAATCA BACH2 1e-28 0.86% 0.54% 18 STGCTGAGTCAT NFE2L2 1e-28 0.27% 0.11% 19 FGCCECEGEC KLF5 1e-28 0.467% 13.29% 20 AGATAASS GATA3 1e-26 3.70% 3.01% 21 ASECGCAATCGC NRF 1e-23 2.47% 1.95% 22 SEGCGAATCGC NRF 1e-23 2.47% 1.95% 23 SEGCGAATCGC NRF 1e-23 3.47% 2.79% 24 SEGCATAGES GATA4 1e-21 2.53% <td< th=""><th>Rank 1 2 3 4 5 6 7 8 9 10</th><th>Motif ASCCAATSX9 X20TQA&TCAXX #ATGA&TCAXXX XATGA&TCAXXX XATGA&TCAXXX XATGACTCAXXX XATGACTCAXXX XATGACTCAXXX XATGACTCAXXX XATGACTCAXXX XATGACCCCCCCCXXX XACCCCCCCCCXXX XACCCCCCCCCXXX XACCCCCCCCCXXX XACCCCCCCCXXX XACCCCCCCCCXXX XACCCCCCCCCXXX XACCCCCCCCXXX XACCCCCCCCXXX XACCCCCCCCCXXXX XACCCCCCCCCXXXXXXXXXXXXXXXXXXXXXXXXXXX</th><th>NAME FOSL1 FOSL2 BATF ATF3 JUN CTCF SP1 JUN TEAD</th><th>P-value 1e-110 1e-87 1e-77 1e-71 1e-70 1e-67 1e-62 1e-56 1e-50 1e-40</th><th>Target Sequences vith Motif 4.65% 2.12% 1.48% 2.30% 2.33% 1.10% 1.23% 5.91% 2.53% 2.26%</th><th>Background Sequences with Motif 3.13% 1.25% 0.81% 1.46% 1.49% 0.57% 0.67% 4.64% 1.77% 1.61%</th></td<>	Rank 1 2 3 4 5 6 7 8 9 10	Motif ASCCAATSX9 X20TQA&TCAXX #ATGA&TCAXXX XATGA&TCAXXX XATGA&TCAXXX XATGACTCAXXX XATGACTCAXXX XATGACTCAXXX XATGACTCAXXX XATGACTCAXXX XATGACCCCCCCCXXX XACCCCCCCCCXXX XACCCCCCCCCXXX XACCCCCCCCCXXX XACCCCCCCCXXX XACCCCCCCCCXXX XACCCCCCCCCXXX XACCCCCCCCXXX XACCCCCCCCXXX XACCCCCCCCCXXXX XACCCCCCCCCXXXXXXXXXXXXXXXXXXXXXXXXXXX	NAME FOSL1 FOSL2 BATF ATF3 JUN CTCF SP1 JUN TEAD	P-value 1e-110 1e-87 1e-77 1e-71 1e-70 1e-67 1e-62 1e-56 1e-50 1e-40	Target Sequences vith Motif 4.65% 2.12% 1.48% 2.30% 2.33% 1.10% 1.23% 5.91% 2.53% 2.26%	Background Sequences with Motif 3.13% 1.25% 0.81% 1.46% 1.49% 0.57% 0.67% 4.64% 1.77% 1.61%
13 GGSSSGGG MAZ 1e-32 17.62% 16.00% 14 SATGACTCAGCA NFE2 1e-31 0.31% 0.13% 15 AAGTAACA FOXA1 1e-31 2.72% 2.08% 16 SCAGGAATQU TEAD2 1e-29 1.95% 1.43% 16 SCAGGAATQU TEAD2 1e-28 0.86% 0.54% 17 ISQCTGAGTCAI NFE2L2 1e-28 0.27% 0.11% 19 AGGCGZGUGGQC KLF5 1e-28 14.67% 13.29% 20 AGATAASSA GATA3 1e-26 3.01% 4.26% 21 AGCGCGAAGU ELF1 1e-24 5.03% 4.26% 22 GECCCCATCCCC NRF 1e-23 2.47% 1.95% 23 SEGGGAATSF TEAD4 1e-22 3.40% 2.79% 24 SEGGCATSS GATA4 1e-21 2.53% 2.02%	Rank 1 2 3 4 5 6 7 8 9 10 11	Motif SQCCAATSXS XSATGASTCAIX SATGASTCAIX XATGASTCAIX XATGASTCAIX SATGASTCAIX SATGASTCAIX SATGASTCAIX SATGASTCAIX SATGASTCAIX SATGASTCAIX SATGASTCAIX SATGASTCAIX SACCCCCCCCCS STGGCATCAIS SXIGGAATYF SXEXSSFGCCCSCTSEIGGS	Name NFY FOSL1 FOSL2 BATF ATF3 JUN CTCF SP1 JUN TEAD CTCFL (BORIS)	P-value 1e-110 1e-87 1e-77 1e-71 1e-70 1e-67 1e-62 1e-56 1e-50 1e-40 1e-37	Target Sequences with Motif 4.65% 2.12% 1.48% 2.30% 2.33% 1.10% 1.23% 5.91% 2.53% 2.26% 2.44%	Background Sequences with Motif 1.25% 0.81% 1.46% 1.49% 0.57% 0.67% 4.64% 1.77% 1.61% 1.79%
14 SATGACTCAGCA NFE2 1e-31 0.31% 0.13% 15 AAGTAAACA FOXA1 1e-31 2.72% 2.08% 16 SCAGGAATGU TEAD2 1e-29 1.95% 1.43% 17 IscTCAGTCA BACH2 1e-28 0.86% 0.54% 18 QTCCTGAGTCAT NFE2L2 1e-28 0.27% 0.11% 19 ACGCIGGCC KLF5 1e-28 14.67% 13.29% 20 ACGTAASSA GATA3 1e-26 3.01% 4.26% 21 ACCCCCATCCCC NRF 1e-23 2.47% 1.95% 23 SEGCGAATSF TEAD4 1e-22 3.40% 2.79% 24 SEGCATASS GATA4 1e-21 2.53% 2.02%	Rank 1 2 3 4 5 6 7 8 9 10 11 12	Motif SSCCAATSXS SSCTQASTCAIX SCTQASTCAIX SCTQASTCAIX SCTQASTCAIX SATGASTCAIX STGGASTCAIX STGGASTCAIX STGGAATY? STSTSASGCCCCCCA SCCACACCCA	Name FOSL1 FOSL2 BATF ATF3 JUN CTCF SP1 JUN TEAD CTCFL (BORIS) KLF4	P-value 1e-110 1e-87 1e-77 1e-71 1e-70 1e-67 1e-62 1e-56 1e-50 1e-40 1e-37 1e-34	Target Sequences with Motif 4.65% 2.12% 1.48% 2.30% 2.33% 1.10% 1.23% 5.91% 2.53% 2.26% 2.44% 3.41%	Background Sequences with Motif 1.25% 0.81% 1.46% 1.49% 0.57% 0.67% 4.64% 1.77% 1.61% 1.77% 2.66%
15 AAGGTAAAÇA FOXA1 1e-31 2.72% 2.08% 16 SCAGGAATGE TEAD2 1e-29 1.95% 1.43% 17 IGCTGASTCA BACH2 1e-28 0.86% 0.54% 18 STCCTCASTCA BACH2 1e-28 0.27% 0.11% 19 SCGCEGGQC KLF5 1e-28 0.467% 13.29% 20 AGATAASSA GATA3 1e-26 3.70% 3.01% 21 GECCCGCAACCI ELF1 1e-24 5.03% 4.26% 22 GECCCCATCCCC NRF 1e-23 2.47% 1.95% 23 SEAGGATSF TEAD4 1e-22 3.40% 2.79% 24 SEAGCATASS GATA4 1e-21 2.53% 2.02%	Rank 1 2 3 4 5 6 7 8 9 10 11 12 13	Motif SSCCAATSXS SSCTOASTCAIX SGTOASTCAIX SGTOASTCAIX XATGASTCAIX SATGASTCAIX SCCCCCCCCCCCC SATGASTCAIX SATGASTCAIX SCCCCCCCCCCCCCC STATASTCAIX SATGASTCAIX SATGASTCAIX SCCCCCCCCCCCCCCC STATASTCAIX SCCCCCCCCCCCCA GCOSSGGG	Name NFY FOSL1 FOSL2 BATF ATF3 JUN CTCF SP1 JUN TEAD CTCFL (BORIS) KLF4 MAZ	P-value 1e-110 1e-87 1e-77 1e-71 1e-70 1e-67 1e-62 1e-56 1e-50 1e-30 1e-34 1e-32	Target Sequences with Motif 4.65% 2.12% 1.48% 2.30% 2.33% 1.10% 1.23% 5.91% 2.53% 2.26% 2.44% 3.41% 17.62%	Background Sequences with Motif 1.25% 0.81% 1.48% 0.57% 0.67% 4.64% 1.77% 1.61% 1.79% 2.66% 16.00%
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17 IsocTGASTCA BACH2 1e-28 0.86% 0.54% 18 QTGCTCAGTCAI NFE2L2 1e-28 0.27% 0.11% 19 AGGCECGGACC KLF5 1e-28 0.467% 13.29% 20 AGATAASS GATA3 1e-26 3.70% 3.01% 21 AGCCCGGAACI ELF1 1e-24 5.03% 4.26% 22 QTGCCGCATCCGC NRF 1e-23 2.47% 1.95% 23 SEGCGATTSP TEAD4 1e-22 3.40% 2.79% 24 SEGCATASS GATA4 1e-21 2.53% 2.02%	Rank 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15	Motif ASCCAATSXS X20TGASTCAIXS X1GASTCAIXS X1GASTCAIXS X1GASTCAIXS X1GASTCAIXS X20CCCCCCCCS ATGASTCAIXS XXIGGAATSF XXIGGAATSF XXISGGATSF QCCACCCA QCSXSGG SATGACTCAICA X1GACTCAICA QCSXIGG SATGACTCAICA X1GACTCAICA X1GACTCAICA X1GACTCAICA X1GACTCAICA X1GACTCAICA X1GACTCAICA X1GACTCAICA X1GACTCAICA X1GACTCAICA X1GACTCAICA X1GACTCAICA X1GACTCAICA X1GACTCAICA	Name NFY FOSL1 FOSL2 BATF ATF3 JUN CTCF SP1 JUN TEAD CTCFL (BORIS) KLF4 MAZ NFE2 FOXA1	P-value 1e-110 1e-87 1e-77 1e-71 1e-70 1e-67 1e-62 1e-50 1e-50 1e-30 1e-37 1e-34 1e-32 1e-31 1e-31	Target Sequences with Motif 4.65% 2.12% 1.48% 2.30% 1.23% 1.10% 1.23% 5.91% 2.53% 2.26% 2.44% 3.41% 1.7.62% 0.31% 2.72%	Background Sequences with Motif 1.25% 0.81% 1.46% 1.48% 0.57% 0.67% 4.64% 1.77% 1.61% 2.66% 1.79% 0.66% 1.00% 0.13% 2.08%
18 QTCCTCAGTCAI NFE2L2 1e-28 0.27% 0.11% 19 AGGCECTGGC KLF5 1e-28 14.67% 13.29% 20 AGATAASS GATA3 1e-26 3.70% 3.01% 21 AGCCCGGAACT ELF1 1e-24 5.03% 4.26% 22 QTCCCGCATCCGC NRF 1e-23 2.47% 1.95% 23 SCACATSG TEAD4 1e-22 3.40% 2.79% 24 SCACATAASS GATA4 1e-21 2.53% 2.02%	Rank 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16	Motif ASCCAATSXS XSATGASTCA3XS XATGASTCA3XS XATGASTCA3XS XATGASTCA3XS SATGASTCA3XS SATGASTCA3XS SATGASTCA3XS SATGASTCA3XS SAGCCCCCCCCS ATGASTCA3XS SCCCCCCCCCCS ATGASTCA3XS SCGCCCCCCCCCS ATGASTCA3XS SCACACCCA GGSSSGGG SATGACTCACCA YAGGAATSZ	Name NFY FOSL1 FOSL2 BATF ATF3 JUN CTCF SP1 JUN TEAD CTCFL (BORIS) KLF4 MAZ NFE2 FOXA1 TEAD2	P-value 1e-110 1e-87 1e-77 1e-70 1e-62 1e-62 1e-56 1e-50 1e-30 1e-34 1e-31 1e-31 1e-31 1e-29	Target Sequences with Motif 4.65% 2.12% 1.48% 2.30% 2.33% 1.23% 5.91% 2.23% 2.26% 2.44% 3.41% 17.62% 0.31% 2.72% 1.95%	Background Sequences with Motif 1.25% 0.81% 1.46% 0.67% 0.67% 0.67% 4.64% 1.77% 1.61% 1.79% 0.66% 1.60% 0.13% 2.08%
19 COCCECCOCC KLF5 1e-28 14.67% 13.29% 20 AGATAASS GATA3 1e-26 3.70% 3.01% 21 AGCCGAAGT ELF1 1e-24 5.03% 4.26% 22 SUCCCCATOCCC NRF 1e-23 2.47% 1.95% 23 SSAGGAATSF TEAD4 1e-22 3.40% 2.79% 24 SCACTAASS GATA4 1e-21 2.53% 2.02%	Rank 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17	Motif SCCAATSXS XSTGASTCAIX STGASTCAIX STGASTCAIX STGASTCAIX STGASTCAIX STGASTCAIX STGASTCAIX SCCCCCCCCS STGASTCAIS XXIGGATYF SXSSSGCC SATGACTCAGCA AAGTAAACA SCAGGAATSI ISCTGASTCA	Name NFY FOSL1 FOSL2 BATF ATF3 JUN CTCF SP1 JUN TEAD CTCFL (BORIS) KLF4 MAZ NFE2 FOXA1 TEAD2 BACH2	P-value 1e-110 1e-87 1e-77 1e-70 1e-67 1e-62 1e-56 1e-50 1e-50 1e-40 1e-31 1e-31 1e-31 1e-29 1e-28	Target Sequences with Motif 4.65% 2.12% 1.48% 2.30% 2.33% 1.23% 5.91% 2.53% 2.26% 2.44% 3.41% 1.7.62% 0.31% 2.72% 1.95% 0.86%	Background Sequencess with Motif 1.25% 0.81% 1.46% 0.67% 0.67% 0.67% 4.64% 1.77% 1.61% 1.79% 2.66% 16.00% 0.13% 2.08%
20 AGATAASS GATA3 1e-26 3.70% 3.01% 21 ACCCGAAQT ELF1 1e-24 5.03% 4.26% 22 SUCCCATCCCC NRF 1e-23 2.47% 1.95% 23 SCRCGAATSF TEAD4 1e-22 3.40% 2.79% 24 SCACTAASS GATA4 1e-21 2.53% 2.02%	Rank 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18	Motif SQCCAATSXS XSTQASTCAIX STTGASTCAIX STGASTCAIX STGASTCAIX SATGASTCAIX SATGASTCAIX SATGASTCAIX SATGASTCAIX SCCCCCCCCCS STGCACCCACCCA CCSCCCCCCC SATGACTCAICA SATGACTCAICA SATGACTCAICA SCCCCCACCCA CCSCCCCCCACCA SATGACTCAICA SCCCCCACCCA SCCCCCCCCACCA SATGACTCAICA SCCCCCACCCA SCCCCCCCCACCA SATGACTCAICA SCCCCCACCCA SCCCCCCCACCA SCCCCCCCCACCA SATGACTCAICA SCCCCCCACCA SCCCCCCCCACCA SATGACTCAICA SCCCCCCCACCA SCCCCCCACCCA SCCCCCCCCACCA SCCCCCCCCACCA SCCCCCCCCACCA SCCCCCCCCACCCA SCCCCCCCCCC	Name FOSL1 FOSL2 BATF ATF3 JUN CTCF SP1 JUN TEAD CTCFL (BORIS) KLF4 MAZ FOXA1 TEAD2 BACH2 NFE2L2	P-value 1e-110 1e-87 1e-77 1e-70 1e-62 1e-50 1e-50 1e-30 1e-34 1e-32 1e-31 1e-29 1e-28 1e-28	Target Sequences with Motif 4.65% 2.12% 1.48% 2.30% 2.33% 2.33% 1.10% 1.23% 5.91% 2.53% 2.44% 3.41% 17.62% 0.31% 2.72% 1.95% 0.86% 0.27%	Background Sequences with Motif 1.25% 0.81% 1.46% 1.49% 0.57% 0.67% 4.64% 1.77% 1.61% 1.77% 1.61% 1.79% 2.66% 10.0% 0.13% 1.43% 0.143% 0.54% 0.11%
21 ACCCCAACT ELF1 1e-24 5.03% 4.26% 22 CCCCATCCCC NRF 1e-23 2.47% 1.95% 23 SCRCCAATCC TEAD4 1e-22 3.40% 2.79% 24 SCACATACC GATA4 1e-21 2.53% 2.02%	Rank 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19	Motif SECCAATEXE SECTORETCREXE SETORETCREXE SETORETCREX	Name FOSL1 FOSL2 BATF ATF3 JUN CTCF SP1 JUN TEAD CTCFL (BORIS) KLF4 MAZ NFE2 FOXA1 TEAD2 BACH2 NFE2L2 KLF5	P-value 1e-110 1e-87 1e-77 1e-70 1e-67 1e-62 1e-50 1e-40 1e-37 1e-34 1e-32 1e-34 1e-32 1e-31 1e-29 1e-28 1e-28 1e-28	Target Sequences with Motif 4.65% 2.12% 1.48% 2.30% 2.33% 2.33% 1.10% 1.23% 2.53% 2.44% 2.44% 2.46% 3.41% 17.62% 0.31% 2.72% 0.86% 0.27% 14.67%	Background Sequences with Motif 1.25% 0.81% 1.46% 1.48% 0.57% 0.67% 4.64% 1.77% 4.64% 1.77% 1.61% 1.61% 1.61% 1.61% 1.61% 1.63% 0.13% 2.68% 0.13% 0.54% 0.11% 13.29%
22 SECCCATCCC NRF 1e-23 2.47% 1.95% 23 SECCCATCC TEAD4 1e-22 3.40% 2.79% 24 SEAGCATACC GATA4 1e-21 2.53% 2.02%	Rank 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20	Motif ASCCAATSXS SECTORETCRESS SETORETCRESS SETORETCRESS SETORETCRESS SETORETCRESS SECOCCCCCCCCS ATGRETCRESS SECOCCCCCCCS SETORETCRESS SECOCCCCCCCS SETORETCRESS SECOCCCCCCCS SETORETCRESS SECOCCCCCCCS SETORETCRESS SECOCCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCCS SETORETCRESS SECOCCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCCS SETORETCRESS SECOCCCCCCCS SETORETCRESS SECOCCCCCCS SETORETCRESS SECOCCCCCCCS SETORETCRESS SECOCCCCCCCS SETORETCRESS SECOCCCCCCCS SETORETCRESS SECOCCCCCCCS SETORETCRESS SECOCCCCCCCS SETORETCRESS SECOCCCCCCCCS SETORETCRESS SECOCCCCCCCCCS SETORETCRESS SECOCCCCCCCCS SETORETCRESS SECOCCCCCCCCS SETORETCRESS SECOCCCCCCCCS SETORETCRESS SECOCCCCCCCCS SETORETCRESS SECOCCCCCCCCS SETORETCRESS SECOCCCCCCCCS SETORETCRESS SECOCCCCCCCCS SETORETCRESS SECOCCCCCCCCCS SETORETCRESS SECOCCCCCCCCCS SETORETCRESS SECOCCCCCCCCS SETORETCRESS SECOCCCCCCCCCS SETORETCRESS SECOCCCCCCCCCS SETORETCRESS SECOCCCCCCCCCS SETORETCRESS SECOCCCCCCCCCCCS SETORETCRESS SECOCCCCCCCCCS SETORETCRESS SECOCCCCCCCCCCCS SETORETCRESS SECOCCCCCCCCCCCCCS SETORETCRESS SECOCCCCCCCCCCCCCCCS SETORETCRESS SECOCCCCCCCCCCCCCCCCCCS SETORETCRESS SECOCCCCCCCCCCCCCCCCCCCCCCCCS SETORETCRESS SECOCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	Name FOSL1 FOSL2 BATF ATF3 JUN CTCF SP1 JUN TEAD CTCFL (BORIS) KLF4 MAZ NFE2 FOXA1 TEAD2 BACH2 KLF5 GATA3	P-value 1e-110 1e-87 1e-77 1e-70 1e-67 1e-62 1e-56 1e-50 1e-40 1e-32 1e-31 1e-31 1e-31 1e-28 1e-28 1e-28 1e-28 1e-28	Target Sequences with Motif 4.65% 2.12% 1.48% 2.30% 2.33% 1.10% 1.23% 5.91% 2.53% 2.26% 2.44% 3.41% 17.62% 0.31% 2.72% 1.95% 0.86% 0.627% 14.67% 3.70%	Background Sequences with Motif 3.13% 1.25% 0.81% 1.46% 0.57% 0.67% 4.64% 1.49% 0.67% 4.64% 1.77% 2.66% 1.61% 1.79% 2.66% 1.60% 0.13% 2.08% 1.43% 0.54% 0.11%
23 SECOGATION TEAD4 1e-22 3.40% 2.79% 24 SCAGATAASA GATA4 1e-21 2.53% 2.02%	Rank 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21	Motif SECCAATEXS SECCAATEXS SETGAETCAIXS SETGAETCAIXS SETGAETCAIXS SETGAETCAIXS SECCCCCCCCCCS SECCCCCCCCCS SECCCCCCCCCS SECCCCCCCCCS SECCCCCCCCCS SECCCCCCCCCS SECCCCCCCCCS SECCCCCCCCCS SECCAECCA SECCCCCCCCCCS SECCCCCCCCCC	Name NFY FOSL1 FOSL2 BATF ATF3 JUN CTCF SP1 JUN TEAD CTCFL (BORIS) KLF4 MAZ NFE2 FOXA1 TEAD2 BACH2 NFE2L2 KLF5 GATA3 ELF1	P-value 1e-110 1e-87 1e-71 1e-70 1e-67 1e-62 1e-56 1e-50 1e-30 1e-31 1e-31 1e-31 1e-31 1e-29 1e-28 1e-28 1e-28 1e-28 1e-28 1e-24	Target Sequences with Motif 4.65% 2.12% 1.48% 2.30% 2.33% 1.10% 1.23% 5.91% 2.53% 2.26% 2.44% 3.41% 17.62% 0.31% 2.72% 1.95% 0.86% 0.27% 1.467% 3.70% 5.03%	Background Sequences with Motif 3.13% 1.25% 0.81% 1.46% 0.57% 0.67% 4.64% 1.49% 0.67% 4.64% 1.77% 2.66% 16.00% 0.13% 2.06% 1.43% 0.54% 0.11% 13.29% 3.01% 4.26%
24 SACATAASS GATA4 1e-21 2.53% 2.02%	Rank 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22	Motif SECCAATEXS SECCAATEXS SETGAETCAIXS SETGAETCAIXS SETGAETCAIXS SETGAETCAIXS SETGAETCAIXS SECCCCCCCCCS SECCCCCCCCCS SECCCCCCCCCS SECCCCCCCCCC	Name NFY FOSL1 FOSL2 BATF ATF3 JUN CTCF SP1 JUN TEAD CTCFL (BORIS) KLF4 MAZ NFE2 FOXA1 TEAD2 BACH2 NFE2 FOXA1 TEAD2 BACH2 NFE2 FOXA1 TEAD2 BACH2 NFE2 FOXA1 TEAD2 BACH2 NFE2 FOXA1 TEAD2 BACH2 NFE2 FOXA1 TEAD2 BACH2 NFE2 FOXA1 TEAD2 BACH2 NFE2 FOXA1 TEAD2 BACH2 BACH2 BACH3 BACH4 BACH3 BACH4 BACH3 BACH4 BACH3 BACH4 BACH4 BACH3 BACH4 BACH	P-value 1e-110 1e-87 1e-71 1e-70 1e-62 1e-62 1e-56 1e-50 1e-30 1e-31 1e-31 1e-31 1e-31 1e-29 1e-28 1e-28 1e-28 1e-28 1e-28 1e-28 1e-24 1e-24 1e-24 1e-24	Target Sequences with Motif 4.65% 2.12% 1.48% 2.33% 1.10% 1.23% 5.91% 2.53% 2.25% 2.44% 2.44% 3.41% 17.62% 0.31% 2.72% 1.95% 0.27% 14.67% 5.03% 2.47%	Background Sequences with Motif 3.13% 1.25% 0.81% 1.46% 1.48% 0.57% 0.67% 4.64% 1.77% 1.61% 1.61% 1.61% 2.06% 1.63% 0.13% 2.06% 1.43% 0.54% 1.3.29%
	Rank 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23	Motif SCCAATSXS XSTQASTCAIX STQASTCAIX STGASTCAIX STGASTCAIX STGASTCAIX STGASTCAIX STGASTCAIX STGASTCAIX SCCCCCCCCS SCCCCCCCCS SCCACCCCA STGASTCAIS STGSSSSSSCCCSSCTSSIGGS SCCACACCCA CGSSGGG SATGACTCAGCA ACACTCAIS SCCACACCA SCCACACACCA SCCACACCACCA SCCACACACCA SCCACACACCA SCCACACACCA SCCACACACCA SCCACACCACCA SCCACACACCA SCCACACACCA SCCACACACCA SCCACACACCA SCCACACACCA SCCACACACCA SCCACACACACACACACACACACACACACACACACACAC	Name NFY FOSL1 FOSL2 BATF ATF3 JUN CTCF SP1 JUN TEAD CTCFL (BORIS) KLF4 MAZ NFE2 FOXA1 TEAD2 BACH2 NFE2L2 KLF5 GATA3 ELF1 NRF TEAD4	P-value 1e-110 1e-87 1e-77 1e-70 1e-62 1e-56 1e-50 1e-30 1e-31 1e-32 1e-31 1e-31 1e-31 1e-29 1e-28 1e-28 1e-28 1e-28 1e-28 1e-24 1e-23 1e-24 1e-23 1e-24 1e-23	Target Sequences with Motif 4.65% 2.12% 1.48% 2.30% 2.33% 1.10% 1.23% 5.91% 2.53% 2.26% 2.44% 2.44% 3.41% 1.762% 0.31% 2.72% 1.95% 0.86% 0.27% 3.70% 5.03% 2.47% 3.40%	Background Sequences with Motif 1.25% 0.81% 1.46% 1.46% 0.57% 0.67% 4.64% 1.77% 1.61% 0.67% 1.79% 2.66% 1.79% 2.66% 1.43% 0.54% 0.11% 13.29% 3.01% 4.26% 1.95% 2.79%



ii	AF Ran	E Known Motifs ^{kMotif}	Name	P-value	% of Target Sequences with Motif	% of Background s Sequences with Motif
	1	ALASIGCCASCTSGTCGSSA	CTCF	1e-177	1.74%	0.68%
	2	ASCCAATESS	NFY	1e-91	4.61%	3.16%
	3	AAAGTAAACA	FOXA1	1e-90	3.34%	2.14%
	4	STREECOCOCOTSSTOR	CTCFL (BORIS)	1e-83	2.88%	1.81%
	5	SCCCCCCCCCC	SP1	1e-64	5.99%	4.57%
	6	SAGATAAGS	GATA1	1e-62	1.92%	1.17%
	7	SETGTTIACAEA	FOXA2	1e-61	2.41%	1.56%
	8	EFCTTATCIS	GATA2	1e-57	2.08%	1.33%
	9	†GGGTGTGTG	KLF5	1e-53	15.24%	13.21%
	10	******	GATA4	1e-50	3.01%	2.13%
	11	AGATAASa	GATA3	1e-45	4.24%	3.23%
	12	SESSCISICE AGACASE	FKD:bHLH half-sites	1e-41	2.97%	2.17%
	13	CAAGATGGCGGC	YY1	1e-39	0.89%	0.50%
	14	GGGGGGGG	MAZ	1e-34	17.67%	15.93%
	15	ROSTICCOS	ELK4	1e-33	5.81%	4.80%
	16	COCCERTANCE	GABPA	1e-31	6.38%	5.34%
	17	RASTICCOSE	ELK1	1e-30	5.74%	4.77%
	18	ACCCGGAAGT	ETS site	1e-28	3.18%	2.49%
	19	FORTICCERE	FLI1	1e-25	8.35%	7.27%
	20	STGITIAC	FOXO1	1e-23	6.96%	6.02%
	21	EGTICCATCGCAA	RFX1	1e-21	1.19%	0.84%
	22	<u><u><u></u></u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u><u></u>	ETV1	1e-21	8.74%	7.75%
	23	<u>QCCACACCCA</u>	KLF4	1e-20	3.34%	2.73%
	24	<u><u><u>A</u><u>e</u><u>C</u><u>C</u><u>G</u><u>G</u><u>A</u><u>A</u><u>G</u><u>I</u></u></u>	ELF1	1e-20	5.09%	4.35%
IV	AL Rank	W3 Known Motifs	Name	P-value	% of Target Sequences with Motif	% of Background s Sequences with Motif
IV	ALI Rank	W3 Known Motifs Motif	Name	P-value 1e-426	% of Target Sequences with Motif 2.13%	% of Background s Sequences with Motif 0.60%
IV	ALI Rank	W3 Known Motifs Motif AGASIGCCAPCTSSTGGSSA SCATGASTGASS	Name CTCF FOSL1	P-value 1e-426 1e-256	% of Target Sequences with Motif 2.13% 3.31%	% of Background s Sequences with Motif 0.60% 1.60%
IV	ALI Rank 1 2 3	W3 Known Motifs Motif AZASZGCC29CT%STGG%S6 XS2TGASTCAZS SSTGASTCAZS	Name CTCF FOSL1 FOSL2	P-value 1e-426 1e-256 1e-237	% of Target Sequences with Motif 2.13% 3.31% 2.28%	% of Background s Sequences with Motif 0.60% 1.60% 0.96%
IV	ALI Rank 1 2 3 4	W3 Known Motifs Motif #EASJGCC89CT%STGG%% #SETGASTCAIS #STGASTCAIS #STGASTCAIS #STGASTCAIS	Name CTCF FOSL1 FOSL2 ATF3	P-value 1e-426 1e-256 1e-237 1e-230	% of Target Sequence: with Motif 2.13% 3.31% 2.28% 3.68%	% of Background Sequences with Motif 0.60% 1.60% 0.96% 1.93%
IV	ALI Rank 1 2 3 4 5	W3 Known Motifs Motif #EASEIGCC8FCT%STGG%84 %SATGASTCAE% %ATGASTCAE% %ATGASTCAE% %ATGASTCAE%	Name CTCF FOSL1 FOSL2 ATF3 JUN	P-value 1e-426 1e-256 1e-237 1e-230 1e-229	% of Target Sequences with Motif 2.13% 3.31% 2.28% 3.68% 1.80%	% of Background s Sequences with Motif 0.60% 1.60% 0.96% 1.93% 0.68%
IV	ALI Rank 1 2 3 4 5 6	W3 Known Motifs Motif SECONFCTSSTGGSSE SECONFCAISE SECONFCAISE SECONFCAISE SECONFCAISE SECONFCAISE SECONFCAISE SECONFCAISE	Name CTCF FOSL1 FOSL2 ATF3 JUN BATF	P-value 1e-426 1e-256 1e-237 1e-230 1e-229 1e-228	% of Target Sequences with Motif 2.13% 3.31% 2.28% 3.68% 1.80% 3.66%	% of Background Sequences with Motif 0.60% 1.60% 0.96% 1.93% 0.68% 1.92%
IV	ALI Rank 1 2 3 4 5 6 7	W3 Known Motifs Motif SECULACCEPCTISTCORSE SECONTONICS	Name CTCF FOSL1 FOSL2 ATF3 JUN BATF CTCFL (BORIS)	P-value 1e-426 1e-256 1e-237 1e-230 1e-229 1e-228 1e-210	% of Target Sequences with Motif 2.13% 3.31% 2.28% 3.68% 1.80% 3.66% 2.92%	% of Background sequences with Motif 0.60% 1.60% 0.96% 1.93% 0.68% 1.92% 1.45%
IV	ALI Rank 1 2 3 4 5 6 7 8	W3 Known Motifs Motif Motif Motif Material States Material States Mat	Name CTCF FOSL1 FOSL2 ATF3 JUN BATF CTCFL (BORIS) JUN	P-value 1e-426 1e-256 1e-237 1e-230 1e-229 1e-228 1e-210 1e-196	% of Target Sequence: with Motif 2.13% 3.31% 2.28% 3.68% 1.80% 3.66% 2.92% 3.95%	% of Background sequences with Motif 0.60% 1.60% 0.96% 1.93% 0.68% 1.92% 1.45% 2.24%
IV	ALI Rank 1 2 3 4 5 6 7 8 9	W3 Known Motifs Motif Motif Motif Material	Name CTCF FOSL1 FOSL2 ATF3 JUN BATF CTCFL (BORIS) JUN ELK4	P-value 1e-426 1e-256 1e-237 1e-230 1e-229 1e-228 1e-210 1e-196 1e-113	% of Target Sequence: with Motif 2.13% 3.31% 2.28% 3.68% 1.80% 3.66% 2.92% 3.95% 5.68%	% of Background Sequences with Motif 0.60% 1.60% 0.96% 1.93% 0.68% 1.92% 1.92% 1.45% 2.24% 4.03%
IV	ALI Rank 1 2 3 4 5 6 7 8 9 10	W3 Known Motifs Motif Motif Motif Matrix M	Name CTCF FOSL1 FOSL2 ATF3 JUN BATF CTCFL (BORIS) JUN ELK4 ELF1	P-value 1e-426 1e-256 1e-237 1e-230 1e-229 1e-228 1e-210 1e-196 1e-113 1e-109	% of Target Sequence, with Motif 2.13% 3.31% 2.28% 3.68% 1.80% 3.66% 2.92% 3.95% 5.68% 5.23%	% of Background Sequences with Motif 0.60% 1.60% 0.96% 1.93% 0.68% 1.92% 1.45% 2.24% 4.03% 3.68%
IV	ALI Rank 1 2 3 4 5 6 7 8 9 10 11	W3 Known Motifs Motif	Name CTCF FOSL1 FOSL2 ATF3 JUN BATF CTCFL (BORIS) JUN ELK4 ELF1 ELK1	P-value 1e-426 1e-256 1e-237 1e-230 1e-229 1e-228 1e-210 1e-196 1e-113 1e-109 1e-103	% of Target Sequence, with Motif 2.13% 3.31% 2.28% 3.68% 1.80% 3.66% 2.92% 3.95% 5.68% 5.23% 5.65%	% of Background Sequences with Motif 0.60% 1.60% 0.96% 1.93% 0.68% 1.92% 1.45% 2.24% 4.03% 3.68% 4.07%
īv	ALI Rank 1 2 3 4 5 6 7 8 9 10 11 12	W3 Known Motifs Motif ALASIGCCAPCTXSTGGX24 XSATGASTCAIX SATGASTCAIX SATGASTCAIX SATGASTCAIX XATGASTCAIX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	Name CTCF FOSL1 FOSL2 ATF3 JUN BATF CTCFL (BORIS) JUN ELK4 ELF1 ELK1 BACH2	P-value 1e-426 1e-256 1e-237 1e-230 1e-228 1e-228 1e-210 1e-196 1e-113 1e-109 1e-103 1e-88	% of Target Sequence: with Motif 2.13% 3.31% 2.28% 3.68% 3.68% 3.66% 2.92% 3.95% 5.68% 5.23% 5.65% 1.29%	% of Background Sequences with Motif 0.60% 1.60% 0.96% 0.68% 1.93% 0.68% 1.92% 1.45% 2.24% 4.03% 3.68% 4.07% 0.65%
īv	ALI Rank 1 2 3 4 5 6 7 8 9 10 11 12 13	W3 Known Motifs Motif ALASIGCCAPCT%STGG%A SATGASTCAIS SATGASTCAIS SATGASTCAIS SATGASTCAIS SATGASTCAIS SATGASTCAIS SATTCASTAIS SATTCAGAS ACCACAAGI SASTTCCGSS ISCTGASTCA ACCAGAAGI SASTCAIS	Name CTCF FOSL1 FOSL2 ATF3 JUN BATF CTCFL (BORIS) JUN ELK4 ELF1 ELK1 BACH2 ETV1	P-value 1e-426 1e-256 1e-237 1e-230 1e-229 1e-228 1e-210 1e-196 1e-113 1e-109 1e-103 1e-88 1e-87	% of Target Sequence: with Motif 2.13% 3.31% 2.28% 3.68% 1.80% 3.66% 2.92% 3.95% 5.68% 5.68% 5.65% 1.29% 9.30%	% of Background Sequences with Motif 0.60% 0.96% 0.96% 0.68% 1.93% 0.68% 1.92% 1.45% 2.24% 4.03% 3.68% 4.07% 0.65% 7.41%
īv	ALL Rank 1 2 3 4 5 6 7 8 9 10 11 12 13 14	W3 Known Motifs Motif ALASIGCCAPCT%STGG%A SATGASTGAIX SATGASTGAIX SATGASTCAIX SATGASTCAIX SATGASTCAIX SATGASTCAIX SATGASTCAIX SATGASTCAIX SATTCCGSA ACCCGAAGI SACCGAAGI ACCGGAAGI	Name CTCF FOSL1 FOSL2 ATF3 JUN BATF CTCFL (BORIS) JUN ELK4 ELF1 ELK1 BACH2 ETV1 ETS	P-value 1e-426 1e-256 1e-237 1e-230 1e-229 1e-228 1e-210 1e-196 1e-113 1e-109 1e-103 1e-88 1e-87 1e-86	% of Target Sequence: with Motif 2.13% 2.28% 3.88% 1.80% 3.66% 2.92% 3.95% 5.68% 5.23% 5.65% 1.29% 9.30% 3.25%	% of Background Sequences with Motif 0.60% 1.60% 0.96% 1.93% 0.68% 1.92% 1.45% 2.24% 4.03% 3.68% 4.07% 0.65% 7.41% 2.17%
īv	ALL Rank 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15	W3 Known Motifs Motif Motif ASABUGCCAPCT&STGGREA SATGASTCAISE SATGASTCAISE SATGASTCAISE SATGASTCAISE SATGASTCAISE SATGASTCAISE ACCGAAGI ASCCGAAGI ASCCGAAGI ASCCGAAGI ASCCAATESE	Name CTCF FOSL1 FOSL2 ATF3 JUN BATF CTCFL (BORIS) JUN ELK4 ELF1 ELK1 BACH2 ETV1 ETS NFY	P-value 1e-426 1e-256 1e-237 1e-230 1e-228 1e-228 1e-113 1e-113 1e-109 1e-103 1e-88 1e-88 1e-87 1e-86 1e-84	% of Target Sequence: with Motif 2.13% 3.31% 2.23% 3.66% 3.66% 2.92% 3.95% 5.63% 5.63% 5.65% 1.29% 9.30% 3.25% 4.40%	% of Background Sequences with Motif 0.60% 1.60% 1.93% 0.96% 1.93% 0.68% 1.92% 1.45% 2.24% 4.03% 3.68% 4.07% 0.65% 7.41% 2.17% 3.14%
īv	ALL Rank 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16	W3 Known Motifs Motif	Name CTCF FOSL1 FOSL2 ATF3 JUN BATF CTCFL (BORIS) JUN ELK4 ELF1 ELK1 BACH2 ETV1 ETV1 ETV1 ETS NFY SP1	P-value 1e-426 1e-256 1e-237 1e-230 1e-229 1e-228 1e-210 1e-196 1e-113 1e-109 1e-103 1e-88 1e-87 1e-86 1e-84 1e-84 1e-83	% of Target Sequence: with Motif 2.13% 3.31% 2.28% 3.66% 3.66% 2.92% 3.95% 5.65% 5.23% 5.65% 1.29% 3.30% 3.25% 4.40% 4.56%	% of Background Sequences with Motif 0.60% 1.60% 0.96% 1.93% 0.68% 1.92% 1.45% 2.24% 4.03% 3.68% 4.07% 0.655% 7.41% 2.17% 3.14% 3.28%
īv	ALL Rank 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17	W3 Known Motifs Motif	Name CTCF FOSL1 FOSL2 ATF3 JUN BATF CTCFL (BORIS) JUN ELK4 ELF1 ELK1 BACH2 ETV1 ETS NFY SP1 FLI1	P-value 1e-426 1e-256 1e-230 1e-229 1e-228 1e-228 1e-210 1e-113 1e-109 1e-103 1e-88 1e-88 1e-88 1e-84 1e-83 1e-81	% of Target Sequence: with Motif 2.13% 3.31% 3.68% 3.68% 3.66% 3.95% 5.68% 5.23% 5.65% 1.29% 9.30% 3.25% 4.40% 4.56% 8.51%	% of Background Sequences with Motif 0.60% 1.60% 0.66% 1.93% 0.68% 1.92% 1.45% 2.24% 4.03% 3.68% 4.07% 0.65% 7.41% 2.17% 3.14% 3.28% 6.77%
īv	ALL Rank 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18	W3 Known Motifs Motif	Name CTCF FOSL1 FOSL2 ATF3 JUN BATF CTCFL (BORIS) JUN ELK4 ELF1 ELK1 BACH2 ETV1 ETS NFY SP1 FL11 GABPA	P-value 1e-426 1e-226 1e-237 1e-230 1e-229 1e-228 1e-229 1e-229 1e-229 1e-229 1e-229 1e-229 1e-229 1e-229 1e-220 1e-229 1e-230 1e-36 1e-86 1e-86 1e-86 1e-86 1e-86 1e-86 1e-86 1e-87 1e-86 1e-86 1e-86 1e-86 1e-86 1e-87 1e-88 1e-86 1e-87 1e-86 1e-86 1e-87 1e-86 1e-86 1e-87 1e-88 1e-87 1e-88 1e-86 1e-87 1e-86 1e-87 1e-86 1e-86 1e-87 1e-86 1e-87 1e-86 1e-86 1e-87 1e-86 1e-86 1e-87 1e-86 1e-86 1e-87 1e-86 1e-86 1e-86 1e-87 1e-86 1e-86 1e-86 1e-86 1e-87 1e-86 1e-87 1e-86 1e-86 1e-87 1e-86 1e-87 1e-86 1e-87 1e-86 1e-87 1e-86 1e-87	% of Target Sequence: with Motif 2.13% 3.31% 3.68% 3.68% 1.80% 3.66% 3.95% 5.68% 5.23% 5.65% 1.29% 9.30% 4.40% 4.40% 4.55%	% of Background Sequences with Motif 0.60% 1.60% 1.93% 0.68% 1.92% 1.45% 2.24% 4.03% 3.68% 4.07% 0.65% 7.41% 2.17% 3.14% 3.28% 6.77% 5.06%
īv	ALL Rank 1 2 3 4 5 6 7 8 9 10 11 2 13 14 15 16 17 18 19	W3 Known Motifs Motif	Name CTCF FOSL1 FOSL2 ATF3 JUN BATF CTCFL (BORIS) JUN ELK4 ELF1 ELK1 BACH2 ETV1 ETS NFY SP1 FL11 GABPA ETS1	P-value 1e-426 1e-256 1e-237 1e-230 1e-229 1e-228 1e-210 1e-103 1e-113 1e-113 1e-113 1e-113 1e-113 1e-113 1e-134 1e-83 1e-83 1e-83 1e-81 1e-77 1e-66	% of Target Sequence: with Motif 2.13% 2.28% 3.68% 3.68% 3.66% 2.92% 3.95% 5.68% 5.68% 5.65% 1.29% 9.30% 3.25% 4.40% 4.55% 6.55% 6.94%	% of Background Sequences with Motif 0.60% 1.60% 0.96% 0.68% 1.92% 1.45% 2.24% 4.03% 3.68% 4.07% 0.65% 7.41% 2.17% 3.14% 2.17% 3.14% 5.06% 5.50%
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īv	ALL Rank 1 2 3 4 5 6 7 8 9 10 11 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 23	W3 Known Motifs Motif	Name CTCF FOSL1 FOSL2 ATF3 JUN BATF CTCFL (BORIS) JUN ELK4 ELF1 ELK1 BACH2 ETV1 ETV1 ETV1 FIN FIN FIN FIN FIN FIN FIN FIN FIN FIN	P-value 1e-426 1e-256 1e-237 1e-230 1e-229 1e-228 1e-210 1e-196 1e-37 1e-36 1e-36 1e-36 1e-37 1e-36 1e-36 1e-36 1e-36 1e-36 1e-66 1e-66 1e-66 1e-62 1e-66 1e-62 1e-66 1e-62 1e-66 1e-62 1e-66 1e-	% of Target Sequence: with Motif 2.13% 3.31% 2.23% 3.68% 3.66% 2.92% 3.95% 5.68% 5.63% 5.65% 4.40% 4.56% 8.51% 6.55% 6.94% 2.85% 7.45% 4.14% 0.86%	% of Background Background 1.60% 0.96% 1.93% 0.68% 1.92% 1.45% 2.24% 4.03% 3.68% 4.07% 0.65% 7.41% 2.17% 3.14% 3.28% 6.77% 5.06% 5.50% 1.96% 6.00% 3.08% 0.43%

key roles in development, motifs for GATA-binding proteins 1-4 (GATA1-4)⁴³⁻⁴⁶ and forkhead box A1 and A2 (FOXA1/A2)⁴⁵⁻⁴⁷ are highly overrepresented in open chromatin at DE and AFE stages (Figure 2Ci-ii). Motifs for many other known developmental factors, including orthodenticle homeobox 2 (OTX2),^{48,49} the T-box factor eomesodermin (EOMES)⁵⁰ and multiple SRY-box transcription factors (SOX2, 4, 6 and 10),⁵¹ are overrepresented only in DE cells (Figure 2Ci). Krüppel-like factor 5 (KLF5) and forkhead box O1 (FOXO1) motifs are highly overrepresented in AFE cells but not DE, consistent with the role of these TFs in airway differentiation and function.⁵²⁻⁵⁴ At the LP3b and ALIw3 stages, among the most overrepresented motifs are members of the Fos and JUN families, which dimerize to form AP-1, a TF with important roles in differentiation.⁵⁵ FOXA1 and KLF5 motifs also remain enriched at these stages consistent with an important role of both factors in airway epithelial biology.^{52,56} Motifs for many ETS factors, including ETS homologous factor (EHF), ETS Proto-oncogene 1, transcription factor (ETS1), ETS transcription factor ELK1 (ELK1) and ETS transcription factor ELK4 (ELK4), become highly overrepresented as the cells differentiate into ALI cultures.

3.2 | The transcriptome through differentiation from iPSC-derived definitive endoderm through to air-liquid interface lung epithelial cell cultures

To compare temporal changes in gene expression, RNA-seq was performed at each stage of differentiation in the ND2.0 and CWRU205 iPSC lines. Gene expression values were quantified using the featureCounts software in the Subread package (r3.1) and normalized for sequencing depth and RNA composition with DESeq2. The PCA plot in Figure 3A shows a good correlation between the samples from the two lines at each stage until the ALI cultures. Normalized count distributions for the two iPSC lines consistently clustered together across all principal components. Once the cells were exposed to ALI conditions, each line remained more similar to itself between 3 and 5 weeks at ALI than to the other line at the same time-point. To accommodate the variance between the two lines at the ALIw3 and ALIw5 stages, results for each time-point were used as biological replicates. Following adjustment for sequencing depth between samples, we identified 8940 differentially expressed genes (P < 0.005) at one or

more stage in the full developmental pathway from DE to ALIw5. A heatmap of the expression profile matrix for the top 1000 differentially expressed genes (DEGs) revealed several of these trajectories including genes most up-regulated in the early stages or those that are ALI-specific (Figure 3B). Among the most significant DEGs, passing a P-value threshold of 0.005, in DE compared to all other stages are Cerberus 1, DAN family BMP agonist (CER1), a cytokine that binds directly to bone morphogenetic proteins and inhibits their activity^{57,58} and Left-Right Determination Factor 1 (LEFTY1), which encodes a transforming growth factor-beta (TGF β) family member (Table 1). LEFTY1 regulates LEFTY2 and NODAL and is required for left-right axis formation during development.⁵⁹ Expression of both CER1 and LEFTY1 at later stages of differentiation has a log₂ fold difference of -1.93 compared to DE. In contrast, among the most significant DEGs in the combined ALIw3 and ALIw5 samples compared to other stages of differentiation are the two subunits of sodium/potassium Na+/K+ ATPase (ATP1A1 and ATP1B1), which together are essential for the active transport of Na+ and K+ across cell membranes (Table 2). Also more highly expressed in ALI cultures is the long non-coding RNA Metastasis-Associated Lung Adenocarcinoma Transcript 1 (MALAT1) and two members of the Serpin family of protease inhibitors SERPINA1 and SERPINA3 (Table 2), which have a log₂ fold change of 1.87, 7.57 and 9.08, respectively, compared to the earlier stages. A key target of SERPINA1 (alpha 1 antitrypsin) is elastase, and defects in the protein are associated emphysema.⁶⁰

To confirm the differentiation protocol was as expected based on previous publications,^{9,10} we examined the expression of a number of known markers by both RT-qPCR and RNA-seq, including the key TFs SOX17 and FOXJ1. As expected, SOX17, an important marker of endoderm differentiation,^{9,10} is >3-fold higher in DE cells compared to AFE, and levels decline further as cells enter lung progenitor stages (Figure 3Ci). Expression of FOXJ1, which marks ciliated epithelial cells in the proximal airway,⁶¹ is maintained through ALIw5 (Figure 3Cii). Other markers for airway epithelial cells that are expressed through ALIw5 include the cell surface-associated mucin MUC4, the gel-forming mucin MUC5B, and the basal cell progenitor marker tumour protein p63 (TP63) (see GEO GSE136859, CompletePath_Counts.csv). Also shown in Figure 3D are immunofluorescence images for the lung and thyroid lineage marker NK2 homeobox 1 (NKX2.1), the FOXA2 transcription factor and the club cell marker secretoglobin family 1A member 1 (SCGBG1A1/CC10) in iPSC ND2.0-derived ALIw5 cells. These

FIGURE 3 Gene expression profiles of iPSC differentiation to ALI. A, PCA for differentially expressed genes at each time-point from DE to ALIw5 in ND2.0 (ND) and CWRU205 (CW) iPSC lines. ND2.0 and CWRU205 iPSC lines are used as biological replicates for each developmental stage from DE to LP3b. Due to substantial divergence between the 2 lines at ALI, week 3 and week 5 ALI for each line are used as replicates for ALI, with i denoting ALIw3 and ii denoting ALIw5 samples. B, Heatmap of 1000 differentially expressed genes, normalized for sequencing depth across all samples. C. Left panels, RT-qPCR of SOX17 (i) and FOXJ1 (ii) normalized to the housekeeping gene PGK1 and shown relative to an average of 3 HBE cultures grown on plastic. * denotes P < 0.05 using an unpaired t test comparing sequential stages. Right panels, corresponding RNA-seq DEseq2 normalized counts. D. Immunofluorescence detection of NXK2.1, FOXA2 and SCGBG1A1 in iPSC ND2.0-derived ALIw5 cultures. For each marker the bottom panels show no primary antibody negative controls (AF488 donkey anti-rabbit IgG and RRX donkey anti-mouse IgG) with nuclei stained with DAPI. Upper panels show AF488 staining of NKX2.1, RRX staining of FOXA2 and SCGBG1A1/CC10 on the left and merged images with DAPI (nuclei) on the right. The magnification is the same for all images and the size bar = $10 \,\mu$ m. A diagram below the fluorescent images represents the multilayered differentiated iPSC cells grown on a permeable membrane and cartoons on the right orientate the images with respect to this diagram



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Gene ^a	log ₂ (FoldChange)	log ₂ (FC)	Wald	P-value	P adjusted ^b
CED1	1.02	0.50	2.00	1 015 02	
CERI	-1.93	0.59	-3.29	1.01E-03	3.40E-03
LEFTY1	-1.93	0.52	-3.72	2.01E-04	8.09E-04
SLC2A3	-5.68	0.66	-8.66	4.61E-18	1.31E-16
LAPTM4B	-1.89	0.46	-4.11	4.03E-05	1.90E-04
ERBB4	-6.29	0.75	-8.37	5.71E-17	1.44E-15
RHOBTB3	-1.73	0.43	-4.00	6.31E-05	2.85E-04
PODXL	-5.24	0.50	-10.51	7.61E-26	4.30E-24
CXCR4	-5.24	0.35	-14.93	2.04E-50	6.81E-48
LARP7	-1.09	0.25	-4.41	1.03E-05	5.49E-05
SFRP1	-4.97	0.36	-13.86	1.06E-43	2.00E-41
SERPINE2	-3.22	0.35	-9.27	1.91E-20	6.82E-19
LIN28A	-9.60	0.32	-30.14	1.50E-199	3.06E-195
OTX2	-6.66	0.59	-11.33	9.59E-30	7.62E-28
TDGF1	-7.13	0.67	-10.66	1.54E-26	9.29E-25
CYP26A1	-5.24	0.73	-7.18	6.74E-13	1.06E-11
SLC2A14	-5.01	0.59	-8.46	2.79E-17	7.28E-16
FZD7	-1.80	0.44	-4.11	3.94E-05	1.86E-04
SCD	-1.26	0.30	-4.13	3.58E-05	1.71E-04
CCNG1	-1.34	0.21	-6.29	3.24E-10	3.62E-09

TABLE 1Most significant DEGsin DE compared to all other stages ofdifferentiation

^aGenes are presented in order of high to low by subtracting the average DEseq2 normalized counts from all other stages from the average DEseq2 normalized counts for DE samples (ND2.0 and CWRU205).

^bAdjusted *P*-value is calculated with the Benjamini-Hochberg procedure.

data suggest that the cells are undergoing successful differentiation to the multiple cell types found within the proximal airway epithelium, though do not exclude the persistence of sub-populations of non-lung fated cells.

Next, to identify key biological processes that alter during the course of differentiation from DE to ALIw5 we performed clustering with an infinite Gaussian process mixture model.⁶² Data in Figure 4A show the GO biological process enrichment analysis with time in the ND2.0 iPSC line. Only 20 of the 29 clusters of genes with terms passing statistical significance (P-value < 0.05) across a wide spectrum of biological functions are shown. Among processes of particular relevance to the lung epithelium are 'epithelial tube branching involved in lung morphogenesis' and several involved in immune and antimicrobial responses. Also of note are examples of the different gene expression profiles shown by individual gene clusters (Figure 4B and Figure S2). Cluster 2 genes show a peak of expression between AFE and LP3 with no transcripts at the start and end of the differentiation pipeline. Cluster 5 genes show no expression at the early stages of differentiation with a gradual increase through AFE and the LP stages, while reaching a plateau at ALI. The gene lists associated with each cluster are shown in Table S2. Among genes in Cluster 5 are some that are involved in innate immunity and the response to pathogens. The genes in Cluster 17 are highly expressed at the start of differentiation and are lost through differentiation, being silenced by ALIw3. Many similar pathways were enriched in clusters identified in the CWRU205 line, although the distributions are different (Figure S3 and S4).

3.3 | Expression of genes involved in key pathways of lung development shows substantial changes between stages of differentiation

To further delineate the biological functions that are significantly altered across individual stages of differentiation, we filtered the DEG data sets for each linear pairwise RNA-seq comparison. Here, we focused on transitions between DE to AFE (Figure 5A) and LP3b to ALIw3 (Figure 5B) due to their relevance to early lung tissue development and airway epithelial cell identity, respectively. DEGs passing a 0.01 adjusted *P*-value threshold were stratified by fold change of ± 2 and then analysed using the PANTHER database (r5.1) and gProfiler (r5.2) to identify biological process enrichment gene ontology terms.

In the transition from DE to AFE, 633 genes were down-regulated (Figure 5Ai) and 1107 genes were up-regulated (Figure 5Aii), illustrating the occurrence of both substantial repression and activation of genes during this key developmental modification. A

Gene ^a	log ₂ (FoldChange)	log ₂ (FC) std. error	Wald statistic	P-value	P.adjusted ^b
ATP1A1	2.80	0.36	7.84	4.35E-15	8.99E-14
MALAT1	1.87	0.40	4.70	2.58E-06	1.56E-05
COL1A2	4.05	0.75	5.39	7.14E-08	5.64E-07
SERPINA1	7.57	0.55	13.66	1.67E-42	3.01E-40
ITGB1	1.95	0.36	5.36	8.29E-08	6.50E-07
CEACAM6	8.09	0.95	8.54	1.38E-17	3.72E-16
B2M	4.12	0.56	7.36	1.77E-13	3.03E-12
H19	2.65	0.82	3.23	1.26E-03	4.13E-03
SPP1	7.52	0.75	10.02	1.23E-23	5.72E-22
SERPINA3	9.08	0.96	9.43	4.19E-21	1.55E-19
CDH1	2.86	0.32	8.88	6.58E-19	2.03E-17
NEAT1	1.81	0.44	4.07	4.60E-05	2.14E-04
KRT8	1.35	0.26	5.17	2.32E-07	1.69E-06
CFTR	4.44	0.38	11.55	7.62E-31	6.46E-29
C3	7.81	0.95	8.22	2.03E-16	4.89E-15
ATP1B1	3.29	0.37	8.79	1.51E-18	4.49E-17
TNFAIP2	6.63	0.55	12.10	9.95E-34	1.04E-31
SPTBN1	1.96	0.28	6.94	3.99E-12	5.67E-11
ALDH1A3	4.83	0.57	8.44	3.22E-17	8.35E-16

^aDEseq2 normalized counts were filtered for those genes with a standard deviation of ≤1000 for all ALI samples (ND2.0 ALIw3, ND2.0 ALIw5, CWRU205 ALIw3 and CWRU205 ALIw5). Using the filtered list, genes are presented in order of high to low by subtracting the average DEseq2 normalized counts from all other stages from the average DEseq2 normalized counts for ALI samples.

^bAdjusted *P*-value is calculated with the Benjamini-Hochberg procedure.

significant enrichment of genes associated with general pathways of cell and organ development was seen in DE cells (Figure 5Ai, in blue and Table S3). Among the DE-specific DEGs are the NANOG homeobox gene (NANOG) and the Nodal Growth Differentiation Factor (NODAL), which both have pivotal roles in early development. Also overexpressed in DE cells are fibroblast growth factor 8 (FGF8), which has broad ranging functions in regulating embryonic development and cell proliferation, migration and differentiation, and Ripply 1 transcriptional repressor 1 (RIPPLY1), which has a critical role in somitogenesis. In contrast, genes more highly expressed in AFE cells are involved in anatomical structure development and, more specifically, tube development (Figure 5Aii, in pink and Table S4). Notably, a profound enrichment of genes involved in epithelial processes such extracellular matrix production and cell adhesion was evident in AFE-specific DEGs. These include Transforming Growth Factor Beta 1 and 2 (TGFB1, TGFB2) and the Laminin Subunits Alpha 1, Alpha 2, Gamma 1 and Gamma 2 (LAMA1, LAMA2, LAMC1, LAMC2). These findings are consistent with the specialization of anterior foregut endoderm and loss of stem cell pluripotency.

Fewer genes showed significant alterations in expression levels between LP3b and ALIw3 (Figure 5B) where 344 are down-regulated (Figure 5Bi) and 445 up-regulated (Figure 5Bii). Genes up-regulated in the transition from LP3b to ALIw3 were enriched for many processes integral to epithelial cell function (Figure 5Bii, green and Table S5). These include defence responses, including innate immunity, inflammatory response and several metabolic pathways. Among the ALIw3-specific genes contributing to these processes are multiple cytokines such as C-C motif Chemokines ligand 2 and 7 (*CCL2* and *CCL7*), interleukin 1 beta (*IL1B*), interleukin receptor like 1 (*IL1RL1*) and interleukin 1 receptor agonist (*IL1RN*), which inhibits IL1B activity, and the antimicrobial peptide defensin beta 4A (*DEFB4A*). Similarly, multiple membrane-associated mucins including *MUC1*, *MUC3A*, *MUC13*, *MUC17* and *MUC21* are significantly up-regulated between LP3b and ALIw3. Genes involved in these pathways are essential for the proper airway epithelial integrity and response to the environment. Many of the pathways up-regulated in the differentiated ALI cultures are impaired by lung disease.

3.4 | Intersecting open chromatin with differential gene expression through differentiation from DE to ALI cultures

Next, we investigated the correlation between open chromatin within 20 kb of gene bodies and differential gene expression from

 TABLE 2
 Most significant DEGs in aggregated ALIw3/w5 compared to all other stages of differentiation



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FIGURE 4 Gene ontology (GO) biological process enrichment analysis identifies key cellular, developmental and immune pathways. A. Dot plot of GO biological process terms enriched in gene expression clusters identified in the differentiation of ND2.0. Clustering was performed using an infinite Gaussian process mixture model. Only trajectories with statistically significant enrichment for a biological process category (20/29) are shown. B. Gene expression trajectories from iPSC to ALIw5 for Clusters 2, 5 and 17. The expression profile for individual genes across the developmental pathway (Table S2) is stratified into cluster models with the aggregate mean and standard deviation plotted for each

cluster mean +/- 2x std. dev.

- individual gene trajectory

- cluster mean



FIGURE 5 Pairwise transition gene ontology process enrichment analysis identifies key developmental and innate immune response processes. Volcano plot of weighted fold change as a function of *P*-values for genes during the transitions from A) DE to AFE and B) LP3b to ALIw3. DEGs passing a -log₁₀ adjusted *P*-value of 2 and log₂ fold change of 2 or -2 determined to be stage specific were processed using gene ontology process enrichment analysis. Biological process terms were chosen by passing a log₁₀ adjusted *P*-value with Bonferroni correction of 4 (Ai), 20 (Aii), 3 (Bi) or 6 (Bii). Notable categories are highlighted in colour for each analysis



FIGURE 6 Differential gene expression correlates with presence of open chromatin. A, Distributions of stage-specific DEGs correlated with ATAC-seq peaks unique to either stage. Genes passing a 0.01 adjusted *P*-value threshold with a peak within 20 kb of the gene body were binned by fold change for each linear pairwise comparison. Comparisons shown are i) DE to AFE, ii) AFE to LP3a, iii) LP3a to LP3b, iv) LP3b to ALIw3. B, RNA-seq DEseq normalized counts for TFs with stage-specific overrepresented motifs (by HOMER) in ATAC peaks i) DE-specific TFs, CRX, EOMES and OTX2; ii) LP3b-ALIw3/5 epithelial selective transcription factors, ETS1, KLF5 and EHF. C. Integrated Genomics Viewer (IGV) browser view of ATAC-seq tracks and RNA-seq read alignments at i) *EOMES* and ii) *EHF* loci comparing DE and AFE, or LP3b and ALIw3 stages, respectively. Arrows indicate stage-dependent changes in chromatin accessibility at gene promoters

the RNA-seq data. DEG results for each linear transition through the differentiation were combined with annotation data for ATACseq peaks specific to each gene. DEGs with at least one peak passing the IDR threshold for each stage were binned by log₂ fold change in the pairwise RNA-seq comparison (Figure 6A). While the overlap analysis shows consistent gene activation and repression around regions of open chromatin, substantially more genes are involved in the early DE to AFE transition (Figure 6Ai), somewhat lower but equivalent numbers are seen at the AFE to LP3a and LP3b to ALIw3 transitions (Figure 6Aii, iv), while many fewer are evident between LP3a and LP3b (Figure 6Aiii). These data are consistent with the major shift in cellular identity from DE to AFE and the more gradual changes through later differentiation and upon transition to ALI.

To reveal more about the TFs driving these changes in cellular identity and differentiation, we examined the expression profiles of specific TFs with important roles in early-stage or late-stage differentiation, for which binding motifs are enriched at peaks of open chromatin (Figure 6B). Among the top enriched motifs in DE cells, 12 are observed only in DE open chromatin (Figure 2C, Figure S1); moreover, 6 are also DE DEGs, including genes encoding the important differentiation factors EOMES and OTX2. Figure 6Bi shows the DEseg2 normalized counts at each state of differentiation for Cone-Rod Homeobox (CRX), EOMES and OTX2. While CRX expression is only seen in DE cells, EOMES and OTX2 show gradual reduction in activity through LP3b and ALI, respectively. Expression of KLF5, for which the binding motif is significantly enriched in peaks of open chromatin in AFE cells and all later stages, gradually increases through post-AFE differentiation, in line with its role in airway epithelial cell differentiation (Figure 6Bii). In contrast to the abundance of ETS1, which is constant through differentiation, expression of EHF, for which the binding motif is only enriched in ALIw3 and ALIw5 cells, reaches maximal expression levels in ALI cells. This is consistent with previous reports of the role of EHF on lung epithelial function and modifying cystic fibrosis lung disease severity.⁶³⁻⁶⁵ To highlight the concordance between the ATAC-seq signal and gene expression levels, we surveyed the open chromatin and RNA-seq profiles at the EOMES and EHF loci. Both genes show some positive correlation of ATAC-seq peak intensity and RNA-seq reads (Figure 6C). Most notable are alterations in ATAC-seq signal adjacent to the gene promoters that correspond to changes in gene expression level, evident from total aligned reads (Figure 6C). For example, loss of the ATAC-seq signal at the EOMES promoter in the transition of DE to AFE correlates with reduced aligned RNA-seq reads in AFE cells (Figure 6Ci). In contrast, gain of open chromatin at the EHF promoter correlates with increased aligned reads at the EHF locus in ALIw3 compared to LP3b (Figure 6Cii).

3.5 | Comparison of iPSC-derived ALI open chromatin and transcriptome with donor-derived HBE-ALI cultures

A critical feature of the iPSC ALI cultures is how well they mirror the transcriptome and functions of ALI cultures generated from adult

human bronchial epithelial cells derived from donor lung tissue. We observed significant differences in both open chromatin (Figure 7A) and gene expression profiles (Figure 7B) between the ALI cultures of different origin. The PCA plots of ATAC-seq peaks show that while biological replicas of iPSC and donor-derived ALI cultures cluster well by origin, they are far from each other, and there is more similarity between the two iPSC lines than the two donors (Figure 7A). With respect to gene expression profiles (Figure 7B), the six replicas of donor-derived HBE-ALI cells cluster well. Though the 2 iPSCderived lines are quite different from the donor-derived HBE-ALI cells there is only a 6% variance between the biological replicates. Technical replicates of each line also cluster well, reflecting robust and consistent culture protocols. Despite the divergence between iPSC- and donor-derived ALI cultures, the top 24 known TF binding motifs overrepresented in the IDR open chromatin peaks of HBE-ALI cells (Figure 7C) show some overlap with iPSC-derived ALI cells (Figure 2Civ and Figure S1C). Key factors regulating normal airway epithelial function including EHF, KLF5, and BTB domain and CNC homolog 2 (BACH2) are among these TFs in common. Unique to donor-derived ALI cells are overrepresented motifs for CCAAT enhancer binding protein (CEBP), BACH1 and E47-like ETS transcription factor 5 (ELF5). The matrix of differential gene expression (Figure 7D) also shows significant differences between the HBE-ALI cultures of different origins. A total of 3394 genes had a ≥2-fold higher expression in donor-derived HBE-ALI, and 3478 genes were more highly expressed in the iPSC-derived ALI cells. A gene ontology process enrichment analysis on these DEGs (Table S6) showed that processes related to cilia and microtubules were the most markedly up-regulated in donor-derived HBE cultures, consistent with failure of the iPSC-derived cells to generate a robust ciliated cell compartment. In contrast, iPSC-derived ALI cultures showed an increase in processes associated with cell surface components, extracellular matrix and cell adhesion. The DEG matrix also illustrates the high degree variability between the two iPSC-derived ALI cultures consistent with data shown in Figure 3A and B. We then examined the relationship between open chromatin profiles and gene expression levels for two genes, Filamin-binding LIM Protein 1 (FBLIM1), which is more highly expressed in iPSC-derived ALI cultures, and Sodium Channel Epithelial 1 Beta Subunit (SCNN1B) (encoding the beta subunit of ENaC), which is more abundant in donor-derived HBE-ALI cells (Figure 7E). Sites of open chromatin at each locus correlated well with differences in gene expression, with more pronounced accessibility at the promoters, introns and downstream elements in the cultures showing higher expression (Figure 7F). CD47 provided a negative control for this analysis, as neither the normalized counts from DESeq2 nor the ATAC-seq profiles (Figure 7E and Figure S5) vary between the cultures.

4 | DISCUSSION

The goal of the experiments described here was to generate unbiased genome-wide data on open chromatin and gene expression



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FIGURE 7 Comparison of ATAC-seq and RNA-seq profiles of donor-derived ALI-HBE and iPSC-derived ALI-HBE cultures. A, PCA plot for ATAC-seq peak distribution comparing primary HBE-ALI cultures from 2 donors (DD076O and DD078O) and 2 samples each from iPSC-derived HBE-ALI cultures CWRU205 and ND2.0. B, PCA plot for RNA-seq gene expression data comparing HBE-ALI cultures from 6 donors and 2 replicas each from ALI-HBE ND2.0 and CWRU205. C, HOMER analysis showing the top 24 known TF binding motifs overrepresented in ATAC-seq peaks from donor-derived HBE-ALI cultures. * and blue font denotes motifs also enriched in top 24 known motifs in iPSC-derived ALI-HBE samples passing a 0.01 adjusted *P*-value threshold. Each donor for primary cells or iPSC-derived cultures was used as a replicate for hierarchal clustering. E, Normalized sequence counts comparing donor-derived and iPSC-derived cells for *CD47* control and *FBLIM1* and *SCNN1B* DEGs. F, UCSC genome browser graphic of ATAC-seq tracks (IDR) for donor-derived and iPSC-derived HBE-ALI cultures at the *FBLM1* and *SCNN1B* loci. Arrows show sites of increased chromatin accessibility in cells with higher gene expression

during the differentiation of iPSCs into lung epithelial cells at ALI, as this model is frequently used for the study of key events in lung disease such as CF. Though careful characterization of the expression of multiple specific marker genes along the differentiation pathway has been performed by several groups,^{7-14,18,20-23} these studies of individual transcripts or their encoded proteins are by definition limited in scope. The definition of key markers has generally relied on extensive data from other mammalian species, most notably the mouse, which is exceptionally well studied (reviewed in⁶⁶). However, the mouse lung has a number of important anatomical and functional differences from the human lung,^{67,68} suggesting that an unbiased review of gene expression profiles in the iPSC-to-ALI differentiation could be valuable. Hence, the genome-wide data reported here are important contributions to understanding the functional genomics of this differentiation pipeline. Several aspects of the differentiation warrant further discussion; although iPSC-derived differentiated cellular models are widely used in medical research, and have facilitated numerous important advances, some pathways of differentiation are more robust than others. Indeed, there are ongoing efforts to optimize the protocols for generating well-differentiated bronchial epithelial cells at ALI from iPSCs. At the time of these experiments, we followed one well-documented protocol of this differentiation pipeline (Figure 1)¹⁰ but are aware that more recent optimizations may enhance the outcome.^{11,13,21,22} Two key guestions warrant further discussion: first, how robust is the differentiation pipeline in different iPSC lines and second, how similar are iPSC-derived HBE-ALI cultures to organ donor-derived HBE-ALI cells.

To address the first question, we investigated the reproducibility of differentiation in terms of variable efficiency and cell heterogeneity in three iPSC cell lines, from two sources. One line repeatedly failed to reproducibly differentiate to the ALI stage and was excluded for further experimentation and analysis. While the other two lines reproducibly differentiated to ALI cultures, one line apparently differentiated much more efficiently than the other. This phenomenon is well illustrated by the divergence between the ALIw3 and ALIw5 transcription profiles of the two donor lines. After 5 weeks of ALI culture, each line was more similar to itself after 3 weeks than to the other culture at 5 weeks. Though ND2.0 ALI cells expressed many markers for proximal lung progenitors (*TP63* and *KRT5* (basal cells), *FOXJ1* (ciliated cells), *ARG2* (goblet cells)), others were expressed at lower levels compared to CWRU205 ALI cells. The latter appeared to provide a more robust model airway epithelium at ALI including expressing markers for secretory cells (SCGB3A2) and goblet cells (MUC5AC), in addition to the cell types seen in ND2.0 ALI cultures. In the context of the divergent differentiation of the CWRU205 and ND2.0 cultures, it is relevant that differentiation of definitive endoderm into airway cells is often associated with the production of nonlung endoderm-derived cells types. These include predominately hepatic-like cells in iPSC-derived proximal airway,^{9,10,18-20} gastric-like cells in iPSC-derived distal airway²⁰ and other endoderm-derived tissue lineages such as pancreas.^{9,10} The differentiated ALI cultures we generated from iPSC cells similarly expressed some markers for non-lung endoderm cells. These include the hepatic markers fibrinogen beta chain (FGB), apolipoprotein A2 (APOA2) and hepatocyte nuclear factor 4A (HNF4A) and to a lesser extent, pancreas as shown by pancreatic and duodenal homeobox (PDX1) expression. The different abundance of non-lung cell types likely contributes to the deviation of expression and open chromatin profiles between the two iPSC-derived ALI cultures.

Directly addressing the second question, there are clear differences between open chromatin and expression profiles of human donor-derived HBE-ALI and ALI derived from iPSCs (Figure 7). Many transcription factors that are expressed in murine lung epithelial cells, and play a role in their differentiation, are highly expressed in both groups of ALI cells in addition to enrichment of their binding motifs in both cell types (Figure 2Civ, Figure S1C, Figure 7C). These TFs include JUN, KLF5 and EHF. However, the binding motifs of other TFs with an important role in the lung epithelium are not enriched in open chromatin from iPSC-derived ALI, despite being similarly expressed in iPSC- and donor-derived ALI cultures. These factors include members of the CEBP family,⁶⁹ BACH1,⁷⁰ NFE2L2 (formerly NRF2) ⁷¹ and ELF5.⁷² The fact that these TFs are present in the cells but are apparently not being selectively recruited to cis-regulatory elements (open chromatin peaks) suggests that critical co-factors required for DNA-binding might be absent or mis-expressed. Alternatively, these TFs may not be appropriately activated by external stimuli in the iPSC-derived ALI cultures, including from other cell types, and so cannot bind DNA. It is also likely that some of the cell types in the donor-derived HBE-ALI cultures that contribute to the motif enrichment signal are either absent or at lower abundance in the iPSC-derived ALI cells.

In conclusion, we provide a detailed molecular characterization of the gene expression repertoire and the activating and repressive TFs that direct the differentiation of iPSCs into lung epithelial cells at ALI. Our data show that though this model to study lung epithelial function in health and disease has many advantages, according to the protocols we used it currently falls short of producing a fully differentiated functional airway epithelium. This may reflect contamination with non-lung fated cells together with a lack of appropriate environmental cues, which may be biochemical (such as appropriate growth factors) or physical (lack of suitable matrix or substrate and stretch forces⁷³), which could result in failure to produce rare but critical cell types. Further higher resolution molecular analysis by single cell RNA-seq and single cell ATAC-seq may reveal the absent components. The application of our analysis pipeline to other protocols for the differentiation of iPSC into lung epithelial cells^{13,14,19,21-^{23,74} would likely be highly informative.}

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CONFLICTS OF INTEREST

The authors confirm that there are no conflicts of interest. The funders had no role in the design of the study, in the collection, analyses or interpretation of the data, in the writing of the manuscript, or in the decision to publish the results.

AUTHOR CONTRIBUTION

Jenny L Kerschner: Conceptualization (supporting); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Writing-original draft (supporting); Writing-review & editing (supporting). Alekh Paranjapye: Conceptualization (supporting); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Writing-original draft (supporting); Writing-review & editing (supporting). Shiyi Yin: Data curation (supporting); Formal analysis (supporting); Investigation (supporting); Methodology (supporting). Dannielle L Skander: Data curation (supporting); Formal analysis (supporting); Investigation (supporting); Methodology (supporting). Gurkan Bebek: Data curation (supporting); Formal analysis (equal); Investigation (supporting); Methodology (supporting); Software (equal). Shih-Hsing Leir: Conceptualization (supporting); Data curation (supporting); Formal analysis (supporting); Investigation (supporting); Methodology (equal). Ann Harris: Conceptualization (lead); Data curation (equal); Formal analysis (equal); Funding acquisition (lead); Investigation (supporting); Methodology (supporting); Project administration (lead); Writing-original draft (lead); Writing-review & editing (lead).

DATA AVAILABILITY STATEMENT

Genome-wide data are deposited at GEO:GSE136859.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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