Repurposing of KLF5 activates a cell cycle signature during the progression from Barrett's Oesophagus to Oesophageal Adenocarcinoma

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Key words: KLF5, Barrett's oesophagus, Oesophageal adenocarcinoma, ERBB2, ATAC-seq

Abstract

Oesophageal adenocarcinoma (OAC) is one of the most common causes of cancer deaths and yet compared to other common cancers, we know relatively little about the underlying molecular mechanisms. Barrett's oesophagus (BO) is the only known precancerous precursor to OAC, but our understanding about the specific events leading to OAC development is limited. Here, we have integrated gene expression and chromatin accessibility profiles of human biopsies of BO and OAC and identified a strong cell cycle gene expression signature in OAC compared to BO. Through analysing associated chromatin accessibility changes, we have implicated the transcription factor KLF5 in the transition from BO to OAC. Importantly, we show that KLF5 expression is unchanged during this transition, but instead, KLF5 is redistributed across chromatin in OAC cells to directly regulate cell cycle genes specifically in OAC. Our findings have potential prognostic significance as the survival of patients with high expression of KLF5 target genes is significantly lower. We have provided new insights into the gene expression networks in OAC and the mechanisms behind progression to OAC, chiefly the repurposing of KLF5 for novel regulatory activity in OAC.

Introduction

Oesophageal cancer is the eight most common cancer worldwide, and its 5-year survival rate of 15% makes it the sixth most-common cause of cancer-related death (Ferlay et al., 2015; Pennathur et al., 2013). A subtype of oesophageal cancer, oesophageal adenocarcinoma (OAC), is the predominant subtype in many Western countries and its incidence is rising rapidly (Coleman et al., 2018). Patients with OAC often present at a late stage with advanced disease (Smyth et al., 2017). The lack of molecular knowledge of OAC, combined with lack of tailored therapies, contribute to the low survival of OAC patients.

The accepted model of OAC development is the progression from an intestinal metaplastic condition of the lower oesophagus, known as Barrett's oesophagus (BO), to OAC through increasing stages of dysplasia (Burke and Tosh, 2012; Spechler and Souza, 2014). Many mutations found in OAC are also present in BO, especially *TP53*, which suggests a stepwise transition to OAC (Ross-Innes et al., 2015; Stachler et al., 2015). Focal amplifications differ as they largely occur in OAC compared to BO (Lin et al., 2012; Stachler et al., 2015; Yamamoto et al., 2016). The amplified genes can be grouped into functional biological pathways with the RAS-ERK signalling pathway (e.g. ERBB2; EGFR; KRAS) and GATA transcription factors (GATA4; GATA6) being the most common (Frankell et al., 2019; Lin et al., 2012; The Cancer Genome Atlas Research Network et al., 2017). The morphology of BO differs from the oesophageal epithelia by the presence of a columnar epithelium and secretory goblet cells, rather than squamous epithelium (reviewed in Spechler and Souza, 2014). Genomic and transcription events have been observed to differ between BO and OAC. Mutations in *TP53* are more frequent in BO from patients that had progressed to OAC (Stachler et al., 2018) and *SMAD4* mutations appear to occur exclusively in OAC, although at a low frequency (Weaver et al., 2014). Increased TGFβ signalling through other SMAD family members, SMAD2/3, promotes growth in OAC cells (Blum et al., 2019). Additionally, increased expression and increased activity of AP-1 transcription factors occurs in the transition from BO to OAC (Blum et al., 2019; Britton et al., 2017; Maag et al., 2017). Despite these studies, the definitive molecular mechanisms of progression to OAC are poorly understood and biomarkers to identify patients at risk of progression are lacking.

Changes to the chromatin landscape have been implicated in many cancers and chromatin accessibility changes during tumourigenesis are a major factor in altering regulatory element activity (Britton et al., 2017; Corces et al., 2018; Davie et al., 2015; Denny et al., 2016; Kelso et al., 2017; Rendeiro et al., 2016; Tome-Garcia et al., 2018; Zhou and Guo, 2018). We recently used Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq)

to ascertain the molecular basis of BO and identified a set of transcription factors that define the BO chromatin landscape and are retained in OAC (Rogerson et al., 2019). Here, we took a similar approach to discover important transcriptional regulators (Fig. 1A) that are specifically operational in OAC and hence contribute to the molecular basis of disease progression from BO to OAC. We compared the open chromatin landscape in BO and OAC patient biopsies and uncovered KLF5 as an important transcriptional regulator that is repurposed to directly drive a cell cycle gene expression signature during the progression of BO to OAC.

Results

Enhanced cell cycle activity defines BO progression to OAC

To begin to understand the molecular events that distinguish OAC form the BO precursor state we first established the differential gene expression profiles between BO and OAC. We analysed public human BO and OAC RNA-seq data (Maag et al., 2017). These samples separate well after principal component analysis (PCA), therefore we retained all samples for further analysis (Supplementary Fig. 1A). Performing differential gene expression analysis, we identified 905 differentially expressed genes between BO and OAC (±1.5x; *Q*-value<0.05; Fig. 1B; Supplementary Table S1). Of these 905 genes, 465 are upregulated in OAC and 440 are downregulated in OAC compared to BO. To validate these findings, we analysed RNA-seq data from our own sample collection (3 BO and 3 OAC). Genes that were upregulated in OAC from the discovery dataset were significantly upregulated in the validation dataset and likewise for downregulated in OAC genes (Fig. 1C). To gain insights into biological pathways behind these differentially expressed genes, we used two approaches. Firstly, Gene Set Enrichment Analysis (GSEA) uncovered two cell cycle associated terms, “G2M checkpoint” and “E2F1 targets”, as the most significant upregulated gene sets in OAC (Fig. 1D). Conversely, “Fatty acid metabolism” and “p53 pathway” as the most significant downregulated gene sets (Supplementary Fig. 1B). Secondly, biological pathway gene ontology analysis of upregulated genes revealed many cell cycle associated terms, such as “Nuclear division”, “Regulation of mitotic cell cycle” and “DNA replication” (Fig. 1E). Example genes such as *CDC25B*, *CENPI* and *E2F1* all showed significant upregulation in OAC compared to BO in both datasets (Supplementary Fig. 1D). Downregulated genes uncovered metabolic associated terms, such as “alcohol metabolic process”, “monocarboxylic acid metabolic process” and “Lipid catabolic process” (Supplementary Fig. 1C). Representative example genes from these pathways such as *IDI1*, *ADH4* and *CIDEC* all show significant downregulation in both datasets (Supplementary Fig. 1E). These initial results indicate a strong upregulation of genes associated with cell cycle processes during the progression from BO to OAC accompanied with the inactivation of genes controlled by the p53 pathway and genes associated with metabolism.

Chromatin accessibility changes in the transition from BO to OAC.

To identify putative transcriptional regulators that may drive the transition to OAC and impact on this enhanced cell cycle profile we analysed the accessible chromatin landscape using ATAC-seq from patient biopsies. To supplement our previous ATAC-seq datasets from BO and OAC patients (Britton et al., 2017; Rogerson et al., 2019), we performed ATAC-seq on two additional OAC biopsies, which were quality-checked and reproducible (Supplementary

Fig. 2A and 2B). We wanted to focus on the differentially expressed genes in OAC compared to BO, therefore we generated a set of accessible regions representing potential regulatory regions that are associated with this set of genes. We took all ATAC-seq peaks from all samples within +/-250 kb of a TSS of a differentially expressed gene (Fig. 2A). After merging so that only unique peaks remained, 35,220 regions were used for further analyses (Supplementary Table S2). We first performed principal component analysis on normalised ATAC-seq signal of all BO and OAC samples to identify differences between samples (Fig. 2B). This led to clustering of all BO samples and clustering of most OAC samples. OAC samples T_003 and T_005 did not cluster with the other OAC samples and were therefore removed from the subsequent differential accessibility analysis. We then carried out differential accessibility analysis between BO and OAC on this peak set (Fig. 2C; Supplementary Table S2). A total of 1495 regions were significantly differentially accessible (±2x; *Q*-value<0.1), the majority of which increased in accessibility (1327/1495). An example gene locus which shows differential accessibility in OAC is centred on *KRT19* (Fig. 2D). Within this locus, both gene promoters (14%; 1/7) and distal regulatory regions (86%; 6/7) gain accessibility in OAC. To assess whether the observed changes of accessibility near differentially expressed genes are common to other OACs, we compared our ATAC-seq data to independent, previously published ATAC-seq datasets from TCGA-ESCA oesophageal adenocarcinoma samples (Fig. 2D, bottom; Fig. 2E; Corces et al., 2018). TCGA-ESCA samples showed similar open chromatin peak profiles and clustered with our OAC samples with the exception of one sample, which clusters with our BO samples (Fig. 2E). The chromatin accessibility profiles nearby genes differentially expressed in OAC are therefore reproducible across patients.

Next we harnessed the differential accessibility data to uncover the identities of transcription factors bound in these regions. *De novo* motif discovery of regions that become more accessible in OAC contain significantly enriched motifs for AP-1, KLF, TBX, NFκB and p53 transcription factor families (Fig. 3A; Supplementary Table S3). AP-1 and KLF were clearly the most frequent motifs in the differential regions and showed the strongest match score for the consensus motif. Regions that showed decreased chromatin accessibility in OAC are enriched in EWSR1-FLI1, ASCL2, GLI2, E2F and ZBTB18 motifs, albeit with relatively low match scores (Supplementary Fig. 2C; Supplementary Table S3). To further assess which transcription factors might be involved in gene expression control, we carried out footprinting analysis on differential accessible regions from our ATAC-seq datasets (Fig. 3B; Bentsen et al., 2019). In differential accessible regions, motifs for KLF (e.g. KLF4, KLF5 and KLF1) and AP-1 (e.g. FOS, JUNB, JUND, JUN and FOSL1/2) transcription factors showed the highest footprinting score in OAC, whereas motifs for homeobox transcription factors (e.g. HNF1A,

HOXA5 and NKX2-5), ARID3A and MEF transcription factors (e.g. MEF2A and MEF2C) showed more footprinting in BO. To provide more evidence for transcription factor occupancy, we then plotted ATAC-seq signal across their motifs. Both FOS (AP-1) and KLF4 (KLF) motifs show a clear increase in footprint depth in OAC, indicative of more transcription factor binding (Fig. 3C). We have previously identified AP-1 as an important regulator in OAC (Britton et al., 2017), but the role of KLF transcription factors in OAC is poorly understood. We therefore focussed on the potential role of KLF transcription factors in the progression of BO to OAC.

KLF5 controls expression of cell cycle genes in OAC

To identify a specific KLF transcription factor that may be bound to these accessible regions, we analysed the expression of individual KLF transcription factors in OAC samples (Fig. 3D). KLF5 was clearly the highest expressed among the KLF family in OAC. KLF5 has been previously implicated in oesophageal squamous cell carcinoma as a tumour suppressor (Tarapore et al., 2013), and has been identified as pro-tumorigenic in gastric cancer via amplifications (Chia et al., 2015). To determine the gene regulatory functions of KLF5, we carried out siRNA mediated knockdowns of KLF5 in OE19 cells, a cell line we identified as having a similar chromatin landscape to OAC biopsies (Rogerson et al., 2019). Knockdown of KLF5 was evident after 3 days siRNA transfection (Supplementary Fig. 3A) and RNA-seq replicates were highly correlative (Supplementary Fig. 3B). Carrying out differential expression analysis identified 4,934 genes (2,637 upregulated and 2,297 downregulated) with significant changes in gene expression (±1.3x; *Q*-value<0.05; Supplementary Fig. 3C; Supplementary Table S4). Biological pathway GO term analysis revealed several enriched terms including “DNA replication” and “Regulation of mitotic cell cycle” for downregulated genes, and terms involving “Oxidative phosphorylation” and “mitochondrial gene expression” for upregulated genes (Supplementary Fig. 3D and Supplementary Fig. 3E). The terms associated with downregulated genes are reminiscent of the terms enriched in genes upregulated in OAC (see Fig. 1). Moreover, GSEA also found similar gene sets: “mitotic spindle”; “G2M checkpoint” and “E2F targets” for downregulated genes and “oxidative phosphorylation”; “xenobiotic metabolism” and “fatty acid metabolism” for upregulated genes (Supplementary Fig. 3F and Supplementary Fig. 3G). Since the genes regulated by KLF5 are involved in similar processes as the genes aberrantly expressed in OAC, we asked whether any of the same genes are in each dataset. 21% (97/465) of the genes upregulated in OAC significantly overlap with those downregulated with siKLF5 (Fig. 3E) and many of these are associated with cell cycle related functions, including genes encoding core cell cycle proteins like CCNE1, E2F1 and various MCM proteins (Supplementary Fig. 3H). Further analysis of the biological pathways enriched within these 97 genes identified very

similar GO terms to those enriched in genes upregulated in OAC compared to BO (Fig. 3F). GSEA also identified the same gene set terms: “G2M checkpoint” and “E2F1 targets” (Fig. 3G).

Next we asked whether these genes are directly regulated by KLF5, and carried out ChIP-seq for KLF5 in OE19 cells. Anti-KLF5 antibodies precipitated KLF5 (Supplementary Fig. 3I) and biological replicates were highly reproducible (Supplementary Fig. 3J). Therefore, we took the overlap of peaks between biological replicates forward for downstream analyses, resulting in 13,542 peaks (Supplementary Fig. 3K; Supplementary Table S5). These peaks are highly enriched in the KLF5 motif, demonstrating the validity of the dataset, and also in AP1(FRA1) and GATA (GATA6) motifs, which we have previously revealed in genome wide studies as implicated in OAC (Britton et al., 2017; Rogerson et al., 2019) (Supplementary Fig. 3L). Focussing on the 97 genes that are upregulated in OAC and also downregulated after KLF5 depletion, 97% have a KLF5 ChIP-seq peak within 0.5 Mb of the TSS and the median distance between a KLF5 ChIP-seq peak and the TSS was 15,604 bp (Supplementary Fig. 3M). This is indicative of direct regulation by KLF5. An example gene is *CDC25B* which harbours multiple KLF5 ChIP-seq peaks surrounding its locus (Fig. 3H). Collectively, these results suggest a direct activator role of KLF5 in controlling cell cycle genes in OAC.

The KLF5 cistrome is reconfigured during the progression from BO to OAC.

Having determined a role for KLF5 in controlling cell cycle associated gene expression in OAC cells, we sought to determine the mechanism through which KLF5 acquires these functions. We first asked whether KLF5 expression changes in the transition from BO to OAC, however no increase in expression was found (Fig. 4A). An alternative mechanism might be through redistributing the binding of KLF5 to different regulatory elements in OAC. We therefore hypothesised that KLF5 is active in both BO and OAC but may regulate specific genes in OAC by binding at different loci.

KLF5 is expressed at similar levels in BO derived CP-A and OAC derived OE19 cells (Supplementary Fig. 4A), therefore we used these cell-lines to model KLF5 activity in BO and OAC. To gain a more comprehensive view of KLF5 function, we performed ChIP-seq for KLF5 in CP-A cells and used spike-in normalisation to better assess differential binding relative to OE19 cells. Anti-KLF5 antibodies precipitated KLF5 in CP-A cells (Supplementary Fig. 4B) and biological replicates were highly reproducible (Supplementary Fig. 4D). We took the overlap of peaks between biological replicates forward for downstream analyses, resulting in 13,526 peaks (Supplementary Fig. 4C; Supplementary Table S5). Motif analysis showed high enrichment of the KLF5 motif further demonstrating the quality of the data

(Supplementary Fig. 4E). KLF5 peaks from CP-A and OE19 cells were merged, generating a combined peak set of 21,353 peaks. Differential binding analysis revealed an altered KLF5 binding profile between CP-A and OE19 cells (Fig. 4B,C; Supplementary Table S5): 8,608 peaks show more binding in OE19 cells (40%); 9,230 peaks are shared between the two cell-lines (44%) and 3,515 show more binding in CP-A cells (16%). An example locus is *CCNE1*, which demonstrates increased KLF5 binding in OE19 cells at the promoter and putative enhancers associated with open chromatin regions (Fig. 4D). Reciprocally, specific binding of KLF5 in CP-A cells is evident at the *NKX3-1* locus and common binding of KLF5 in both CP-A and OE19 cells is evident at the *JAG1* locus (Supplementary Fig. 4F).

We next asked whether the regions that exhibit differential binding are enriched for specific transcription factor motifs. Regions that are bound by KLF5 in OE19 cells are enriched in motifs for KLF, GATA, Forkhead, AP-1 and TCF transcription factors, whereas regions bound by KLF5 in CP-A cells are enriched for a different set of motifs with, TEAD, RUNX and p53 transcription factor families in addition to KLF and AP-1 motifs detected (Fig. 4E; Supplementary Table S6). These results are in-keeping with our previous work showing AP-1 and GATA6 functionality in OAC (Britton et al., 2017; Rogerson et al., 2019). Regions specifically bound by KLF5 in OAC cells also exhibited increased accessibility in OE19 cells and importantly, accessibility is also elevated around these binding sites in OAC tissue (Supplementary Fig. 4G). These findings are therefore consistent with a broad role of KLF5 in OAC.

Since oncogenic events during the progression from BO to OAC are poorly understood, we decided to focus on regions that acquire KLF5 binding in OE19 cells. To relate specific KLF5 binding events to gene expression changes, we took the set of 97 genes that are upregulated in OAC and downregulated by KLF5 depletion (ie activated by KLF5; Fig. 3E) and found that there are 371 OE19-specific KLF5 binding peaks within a 0.5 Mb locus centred on the TSS. To further explore how KLF5 activates these genes in OAC, we assessed the transcription factor binding motif distribution (identified in Fig. 4E) within this set of OE19-specific KLF5 peaks. We detected KLF5 binding motifs in 257/371 of these regions, and strikingly, 56% (145/257) of the peaks also house a mixture of FOXA, AP-1, GATA and TCF motifs, in addition to the KLF motif (Fig. 4F; Supplementary Table S7). This suggests that KLF5 functions in a combinatorial manner with these other transcription factors to activate gene transcription during progression from BO to OAC. However, a large portion of these peaks (44%) contain only a KLF motif suggesting a more independent role for KLF5 in these regions (Fig. 4F). To test whether these motif enrichments reflect transcription factor binding, we integrated our ChIP-seq data of transcription factors active in OAC (GATA6 and HNF4A; Rogerson et al, 2019), with KLF5 binding data. Since only GATA motifs are

enriched in these regions we would expect co-binding with GATA6 and not HNF4A. We therefore compared ChIP-seq profiles for these transcription factors, and see extensive co-binding of GATA6 at these sites but no evidence of co-binding with HNF4A (Supplementary Fig. 4H). Finally, the predicted target gene co-regulation by KLF5 and GATA6 was validated by depletion of each factor in OE19 cells, which leads to a large significant overlap in downregulated genes (Fig. 4G). However, this co-regulated gene set contains only two of the cell cycle associated genes regulated by KLF5, suggesting that this combination of transcription factors is not directly involved in controlling this process.

To establish whether the 371 KLF5 bound regions that are associated with KLF sensitive genes are relevant to OAC, we turned back to our ATAC-seq data and clustered the data to reveal two clusters. One set of regions is already partially open in CP-A cells that increase in accessibility in OE19 cells (cluster 1) and another set are closed in CP-A cells and become more accessible (cluster 2) (Fig. 4H, Supplementary Fig. S4I, left). Importantly, the same pattern of accessibility is evident using ATAC-seq signal from BO and OAC tissue (Fig. 4H, Supplementary Fig. S4I, right). To identify any potential differences between these clusters, we performed motif analysis (Supplementary Fig. S4J; Supplementary Table S8). The most common motif in both clusters were KLF motifs and the most striking difference is the large proportion of AP1 motifs specifically associated with cluster 1 suggesting a potential role for AP1 in priming binding of KLF5 to these regions.

Together, these results indicate an altered DNA binding profile for KLF5 in BO and OAC, and this altered binding is associated with chromatin opening. This altered binding profile for KLF5 in OAC reflects a direct role in controlling genes involved in cell cycle.

KLF5 converges with ERBB2 on cell cycle gene regulation and controls cell proliferation in OAC.

Our results indicate a role of KLF5 in controlling increased cell cycle gene expression in OAC, however it is unclear how this relates to genetic events that potentially impact on the same process. Genomic amplifications in signalling receptors are common in OAC, such as *ERBB2* (32% OAC have an *ERBB2* amplification; The Cancer Genome Atlas Research Network et al., 2017) and occur during the transition from BO to OAC (Stachler et al., 2015). As the ERK pathway is implicated in promoting cell proliferation and is controlled by ERBB2, we investigated whether ERBB2 signalling impacts on KLF5-mediated gene regulatory events. First, we sought evidence for a link with transcription factor activity, and performed ATAC-seq on OE19 cells to investigate whether depletion of ERBB2 could alter chromatin accessibility. ERBB2 was efficiently reduced after 72 hours of siRNA treatment and

phosphorylation of downstream targets (ERK and AKT) was reduced (Supplementary Fig. 5A). ATAC-seq data were reproducible and good quality (Supplementary Fig. 5B and Fig. 5D). We performed differential accessibility analysis, which identified 717 regions with decreased chromatin accessibility and 733 regions with increased accessibility (Fig. 5A; Supplementary Table S9). *De novo* motif analysis of the regions that exhibit reduced chromatin accessibility following ERBB2 depletion, revealed that the majority contain AP-1 binding motifs as expected from the established connections between ERK pathway signalling and AP1 transcription factors. However, the binding motif for KLF transcription factors was also detected, albeit in a subset of the regions (Fig. 5B; Supplementary Table S10). We then used our KLF5 ChIP-seq dataset from OE19 cells to validate KLF5 binding at regions with reduced chromatin accessibility following ERBB2 depletion (Supplementary Fig. 5E). These regions are relevant in the context of OAC as they also show increased chromatin accessibility in OAC tissue compared to BO (Fig. 5C). The convergence of ERBB2 signalling on KLF5 transcription factor activity suggested that they might also converge on the same genes. We therefore carried out RNA-seq in OE19 cells (which contain an amplification of the *ERBB2* locus; Dahlberg et al., 2004) treated with siRNA against ERBB2. The RNA-seq data were highly reproducible (Supplementary Fig. 5C) and the expression of the majority of the directly activated KLF5 target genes was reduced upon ERBB2 knockdown (Fig. 5D). Most of these common target genes are cell cycle related. These results therefore indicate that ERBB2 and KLF5 converge on a similar set of regulatory regions to drive the expression of cell cycle regulatory genes.

Finally, we assessed whether defective KLF5-driven cell cycle gene regulation led to proliferative defects in OAC cells. We first depleted KLF5 in OE19 cells using siRNA which resulted in the reduction of KLF5 protein (Supplementary Fig. 5F), and the growth of cells was significantly impeded after siKLF5 treatment (Fig. 5E). Second, we validated this growth defect by using CRISPR interference technology. Stable transfection of dCas9-KRAB and subsequent transfection of sgRNAs targeting the promoter of KLF5 (sgKLF5) into OE19 cells resulted in the reduction of KLF5 protein levels (Supplementary Fig. 5G). CRISPRi knockdown of KLF5 also significantly reduced the growth of OE19 cells (Fig. 5F), mirroring the result with siKLF5. We further explored the role of KLF5 in cell growth and cell cycle progression by performing similar assays while perturbing KLF5 target genes (*CCNE1*, *CDC25B*, *KIF14*, *CLSPN* and *NR4A1*). All these genes showed significant reductions in expression upon siRNA treatment (Supplementary Fig. 5H). The growth of OE19 cells was significantly reduced with the treatment of siRNA against *CCNE1*, *KIF14* and *CLSPN* (Fig. 5G). Knockdown of these genes also significantly altered cell cycle patterns, particularly knockdown of *CLSPN* which induced a prominent S-phase block (Supplementary Fig. 5I).

These results provide more evidence for the role of KLF5 in the growth of cells and highlight the role of KLF5 target genes in this phenotype.

To assess whether the expression of KLF5 and its target genes has any clinical relevance, we sourced OAC expression and survival data (The Cancer Genome Atlas Research Network et al., 2017) and plotted a survival of patients with high and low expression (± median) of KLF5 itself and KLF5 target genes up to 24 months (Fig. 5H). Those with a higher expression of KLF5 showed no difference in patient survival, whereas patients with high target gene expression exhibited a significantly lower survival rate compared to those with low expression. This result is in keeping with the hypothesis that it is the activation of KLF5 target genes by its redistribution across chromatin, rather than its expression level that is important. It is noteworthy that CLSPN expression alone is predictive of increased patient survival (Fig. 5H) and its enhanced expression in OAC compared to BO makes this a useful potential biomarker (Fig. 5I).

Collectively, these results confirm the functional role of KLF5 in cell cycle control in OAC and convergence of action with the ERBB2 signalling pathway. This is clinically important as patients with highly expressed KLF5 target genes have a worse prognosis that those without.

Discussion

Genome sequencing efforts of patients with BO and OAC have provided insights into the molecular causes of BO and OAC and show the mutational relationships between these disease states (Ross-Innes et al., 2015; Stachler et al., 2015). This has provided evidence for a model of OAC developing from BO. The molecular mechanisms involved in progression to OAC are poorly understood, however BO offers a therapeutic window of opportunity to identify those more at risk of OAC development. In addition to genetic events, epigenetic changes and alterations to the chromatin landscape are also likely to play an important role in disease progression. Here, we demonstrate that there are marked changed in chromatin accessibility and associated gene expression, indicating active changes at the chromatin level during carcinogenesis. One of the major contributing factors to this change is the transcription factor KLF5. KLF5 is re-purposed in OAC cells and its chromatin binding profile is massively rewired to drive increased expression of cell cycle associated genes (Fig. 5J).

Cell cycle deregulation is one of the key hallmarks of cancer (Hanahan and Weinberg, 2011) and here we uncovered a cell cycle gene expression signature, comprised of genes that are overexpressed in OAC. Recent research identified the cell-cycle as a perturbed pathway in OAC and suggested the possibility of CDK4/6 inhibitors as a therapeutic treatment (Frankell et al., 2019; Mourikis et al., 2019). We have previously uncovered a deregulated FOXM1 regulatory network active in OAC, a key regulator of late cell cycle gene expression (Wiseman et al., 2015). By integrated ATAC-seq data to identify upstream regulators of this signature, we also uncovered AP-1 and KLF5 as putative transcription factors in this process. We have previously identified AP-1 as an important factor in OAC (Britton et al., 2017) and others have shown an increase in AP-1 family transcription factors between non-dysplastic BO and low-grade dysplastic BO (Maag et al., 2017). What is less clear is the role of KLF5 in the progression of BO to OAC. KLF5 has been shown to have a tumour promoting function in pancreatic (He et al., 2018) and basal-like breast cancer (Qin et al., 2015). KLF5 is also frequently amplified in gastric cancer (Chia et al., 2015; Zhang et al., 2018) and has recently been shown to regulate gene expression in OAC in combination with other transcription factors, GATA6, ELF3 and EHF (Chen et al., 2019). This was recently reinforced by a recent study that identified KLF5 as a master transcription factor on which OAC cell-lines were dependent (Reddy et al., 2019). Paradoxically, KLF5 has been shown to have a tumour suppressor role in oesophageal squamous cell carcinoma (Tarapore et al., 2013) and breast cancer (Chen et al., 2002). The expression of the related protein, KLF4, together with three other genes, was able to stratify OAC from BO, albeit KLF4 expression is reduced in progression from BO to OAC (Maag et al., 2017).

Previous studies have begun to suggest a role for KLF5 in cell cycle control. For example, KLF5 binds to a *CCNE1* promoter proximal element in bladder cancer cells (Pattison et al., 2016) and KLF5 increases the expression of *Ccnb1* and *Mcm2* downstream of oncogenic Ras in fibroblasts (Nandan et al., 2005). Here, we provide evidence that KLF5 exhibits a widespread role; directly controlling cell proliferation through activation of cell cycle associated genes. We also show that reduction of KLF5 levels, or several of its target genes, in OAC cells impairs growth. Indeed, this is exemplified by *CLSPN* which may have therapeutic potential as its gene product, Claspin, has recently been shown to have a broader role in cancer cell viability by protecting cancer cells from replication stress (Bianco et al., 2019). KLF5 directly binds and regulates core cell cycle genes e.g. *CDC25B*, *CCNE1* and *MCM2*, some of which are cell cycle transcription factors e.g. E2F1, MYBL2, thus providing a mechanism for propagating its effects on cell cycle control. We also show KLF5 expression is almost unchanged between BO and OAC. By profiling KLF5 chromatin binding in BO and OAC cells, we have demonstrated an altered KLF5 binding profile. The regions bound by KLF5 specifically in OAC cells are enriched in motifs for several transcription factors, including the GATA family which suggests a combinatorial regulatory code. This is in keeping with our finding that there is extensive overlap between the binding of KLF5 and GATA6 which is reinforced by recent studies that show that KLF5 binds with GATA6 in OAC (Chen et al., 2019) and gastric cancer (Chia et al., 2015).

The overlap in regulatory potential with GATA6 provides a plausible link to one of the major genetic events that drive the BO to OAC transition. Our work also suggests a link to the other major pathway that is activated through gene amplification, the ERBB2-driven RAS-ERK pathway. Knockdown of ERBB2 reduced the expression of many KLF5 target genes and KLF5 motifs were found at regions with reduced chromatin accessibility upon ERBB2 knockdown. It is possible that ERBB2 and other RTK-driven pathways might contribute to KLF5 redistribution in OAC, but this needs further investigation. The signalling pathways are more unclear in the context of BO, the precancerous precursor. We see enrichment of the TEAD motif only in CP-A cells and not OE19 cells, suggesting that KLF5 may be operating through the Hippo signalling pathway in BO. In other contexts, KLF5 has been shown to cooperate with TEAD transcription factors, downstream of YAP/TAZ (Wang et al., 2015) and KLF5 is stabilized by YAP in breast cancer cells (Zhi et al., 2012). Further work is needed to substantiate these links in BO.

In summary, we have used integrative analysis of RNA-seq and ATAC-seq from BO and OAC patient samples to uncover a cell cycle signature regulated by KLF5. Using a multi-omics approach, we found an oncogenic role of KLF5 in OAC, a transcription factor that has not been shown to be mutated, or amplified and or over-expressed in OAC. This study

highlights the power of supplementing expression data with genome-wide chromatin profiling methods such as ATAC-seq. This provides molecular insights into the mechanisms by which BO progresses to OAC and identifies a signature of transcription factor gene targets that have potential prognostic significance and could be used as biomarkers in the clinic.

Methods

Cell lines, cell culture conditions and patient biopsies

OE19 and CP-A cells were purchased from ATCC and tested negative for mycoplasma. OE19 cells were maintained in Gibco™ RPMI 1640 (ThermoFisher, 52400) supplemented with 10% Gibco™ fetal bovine serum (ThermoFisher, 10270) and 1% Gibco™ penicillin/streptomycin (ThermoFisher,15140122). CP-A cells were cultured in keratinocyte serum free media (ThermoFisher, 17005042) supplemented with 5 µg/L EGF (ThermoFisher, 10450-013), 50 mg/L bovine pituitary extract (ThermoFisher, 13028014) and 10% Gibco™ fetal bovine serum (ThermoFisher, 10270) and 1% Gibco™ penicillin/streptomycin (ThermoFisher,15140122).

OE19-dCas9-KRAB stable cells were generated by transfecting $1x10^6$ OE19 cells with 7.5 µg Cas9 plasmid with guides targeting the *AAVS1* locus (addgene #42230; 5'-GGGGCCACTAGGGACAGGAT-3') and 7.5 µg donor plasmid (pAAVS1-Puro-DNR; Origene GE100024) containing doxycycline inducible dCas9-KRAB with Fugene HD (Promega, E2311), as per manufacturer's instructions. After 7 hours, media was replaced and supplemented with 7.5 µM RS-1 (Sigma-Aldrich, R9782) and 1 µM SCR7 pyrazine (Sigma-Aldrich, SML1546), to promote homologous recombination and to inhibit non-homologous end joining respectively. Media was changed the next day and cells were selected with puromycin (0.75 µg/ml) for 14 days. Selected colonies were re-plated to grow single clones and clones screened for dCas9-KRAB protein expression by immunoblotting. OE19-dCas9-KRAB cells were cultured with 100 ng/mL doxycycline (Sigma-Aldrich, D3447) to induce dCas9-KRAB.

Fresh frozen OAC 2 mm biopsies were obtained by consenting patients undergoing endoscopy. Tissue collection granted by the ethics committee of Salford Royal NHS Foundation Trust (04/Q1410/57). Patient consent was obtained in written form and signed by the patient and doctor.

Protein extraction and immunoblotting

Cells were lysed directly in RIPA buffer and incubated on ice for 5 minutes. The lysate was then sonicated in a water bath sonicator (Diagenode Bioruptor) for 5 minutes, 30 seconds on/off and protein quantified using Pierce™ BCA Assay Kit (ThermoFisher, 23227). Lysates were supplemented with SDS-PAGE loading dye to a final concentration of 1x and boiled for 10 minutes. Equal amounts of protein were separated on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane (GE life sciences, 1060002) using a Pierce™ Power Station (ThermoFisher). Membranes were blocked using Odyssey® blocking buffer (Licor, 927-40000) and then incubated with antibodies against KLF5 (abcam, ab137676),

Tubulin (Sigma-Aldrich, T9026), ERBB2 (ThermoFisher, MA5-14057), phospho-ERBB2 (Cell Signalling Technologies, 6942S), AKT (Cell Signalling Technologies, 2920S), phospho-AKT (Cell Signalling Technologies, 9106S), ERK1/2 (Cell Signalling Technologies, 4695S) or phospho-ERK1/2 (Cell Signalling Technologies, 9106S) overnight at 4°C. Membranes were incubated with IRDye secondary antibodies (Licor, 925-32212, 925-32213) and imaged using a Li-Cor Odyssey scanner.

RT-qPCR

RT-qPCR was carried out using QuantiTect SYBR® Green RT-PCR Kit (Qiagen, 204243) using the primer pairs detailed in Supplementary Table S11. Relative gene expression was calculated using the ΔΔCT method relative to levels of *GAPDH* mRNA.

siRNA and sgRNA transfection

200,000 cells were plated on a 6-well plate and incubated for 24 hours. 100 pmol either control non-targeting (siNT; Dharmacon, D-001810-10-0020), siKLF5 (Dharmacon, L-013571-00-0005), or siERBB2 (Dharmacon, L-003126-00-0005) SMARTpool siRNA was transfected per well using Lipofectamine™ RNAiMAX (Thermofisher, 13778150) as per the manufacturer's instructions and incubated for 72 hours. Modified full length sgRNAs were designed using E-CRISP (Heigwer et al., 2014; available at http://www.e-crisp.org/E-CRISP) using the *KLF5* TSS (±200 bp) as input and obtained from Synthego. 9 pmol of either control non-targeting sgRNA (5'- GUAAGGCUAUGAAGAGAUAC-3') or sgKLF5 pool (5'-GUGCGCUCGCGGUUCUCUCG-3'; 5'- AGGACGUUGGCGUUUACGUG-3'; 5'-GCGUCAAGUGUCAGUAGUCG-3') was transfected per well using Lipofectamine™ RNAiMAX (Thermofisher, 13778150). Media was changed after 72 hours for longer treatments.

RNA extraction, RNA-seq processing and analysis

RNA was extracted from cells using a RNeasy RNA extraction Kit (Qiagen, 74136) and quality checked using Nanodrop 1000 (ThermoFisher). Paired-end RNA-seq libraries were generated using TruSeq stranded mRNA library kit (Illumina) and sequenced on a HiSeq 4000 platform (Illumina) by the University of Manchester Genomic Technologies Core Facility. Reads were trimmed using Trimmomatic v0.32 (Bolger et al., 2014) quality checked using FastQC (available at: http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) and aligned to RefSeq transcript annotation of GRCh37 (hg19) using STAR (Dobin et al., 2013). Reads aligned to chromosomes 1-22 and chromosome X were retained. The Cufflinks package v2.2.1 (Trapnell et al., 2012) was used to calculate gene expression levels using Cuffnorm, and to analyse differential gene expression using Cuffdiff. Default parameters were used in both instances. Significant gene expression changes were defined by a fold

change of ±1.3 and a *Q*-value of <0.05. For ERBB2 knockdown experiments, counts for genes were determined using featureCounts (Liao et al., 2014). Log_2 transformed counts were obtained using DESeq2 variance stabilising transformation (VST) function.

Crystal violet assay

200,000 cells were plated on a 6-well plate and siRNA/sgRNA treatment started after 24 hours incubation. At specific time-points after treatment plates were washed with PBS and fixed with 4% paraformaldehyde for 10 minutes. Plates were then washed twice with PBS and kept at 4°C until. Cells were then stained by first incubating plates at room temperature for 10 minutes in 0.1% Triton X-100 with gentle shaking and then incubated at room temperature for 30 minutes in 0.1% crystal violet (Sigma-Aldrich, HT90132) with gentle shaking. Plates were extensively washed with water multiple times and left to dry. The dye was solubilised with 10% acetic acid for 10 minutes with gentle shaking and absorbance was read at 590nm. Values for siNT at each time-point were used as 100% growth.

Propidium iodine staining assay

Cells were trypsinised and collected as a single cell suspension, washed with cold PBS, then fixed in 70% ethanol and stored at -20°C for at least 2 hours. Cells were then resuspended in staining solution (50 µg/mL propidium iodide (Sigma, P4170), 100 µg/mL RNase (Sigma, R4642)) and incubated at room temperature for 30 minutes. Cells were analysed by the University of Manchester Flow Cytometry Core Facility on a LSRFortessa. Percentages of cells in each cell cycle phase were calculated using ModFit LT (http://www.vsh.com/products/mflt/).

ATAC-seq, processing and analysis

Patient samples were processed as previously described (Britton et al., 2017) and omni-ATAC-seq was performed as previously described (Corces et al., 2017). ATAC-seq libraries (~8 per lane) were sequenced on a HiSeq 4000 platform (Illumina) by the University of Manchester Genomic Technologies Core Facility. Reads were quality checked using FastQC (available at: http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) and aligned to GRCh37 (hg19) using Bowtie2 v2.3.0 (Langmead and Salzberg, 2012) with the following options: -X 2000 –dovetail. Unique reads (>q30) aligned to chromosomes 1-22 and chromosome X were retained. Peaks were called using MACS2 v2.1.1 (Zhang et al., 2008) with the following parameters: -q 0.01 –nomodel --shift -75 --extsize 150 -B –SPMR. Peaks called from individual samples were merged using mergePeaks.pl (using d=250 parameter) from the HOMER package v4.9 (Heinz et al., 2010) and resized to peak summit ±250 bp to generate a peak set on which to perform differential accessibility analyses. Amplifications in patient biopsies were removed as described previously (Denny et al., 2016) using a fold change of

16. bedGraph files were converted into BigWig files using bedGraphtoBigWig and visualised in the UCSC Genome Browser (Kent et al., 2002).

For comparing BO and OAC ATAC-seq, the Cufflinks package v2.2.1 (Trapnell et al., 2012) was used to calculate chromatin accessibility levels using Cuffnorm, and differential chromatin accessibility was analysed using Cuffdiff. Default parameters were used in both instances. Significant chromatin accessibility changes were defined as a fold change of ±2 and a *Q*-value of <0.1.

For ERBB2 knockdown experiments differential accessibility was calculated using DESeq2 (Love et al., 2014). Alignment files of biological repeats were combined, peaks recalled and peaks from both conditions were then merged using using mergePeaks.pl (using d=250 parameter) from the HOMER package v4.9 (Heinz et al., 2010) and resized to peak summit ±250 bp. featureCounts from the SUBread package (Liao et al., 2014) was used to count reads within peaks from ATAC-seq samples and these were used an input for DESeq2 to calculate differential binding using default settings. A linear fold change of ±2 and a Q-value of <0.05 were used as a cut-off for further analyses.

ATAC-seq data visualisation

ATAC-seq fragment size was visualised using a custom python script. Correlation plots between technical replicates were visualised using multiBamSummary and plotCorrelation from the deepTools package (Ramírez et al., 2016). Tag density plots and heatmaps were also generated using computeMatrix and plotProfile or plotHeatmap tools from the deepTools package. ATAC-seq counts were also visualised using Morpheus (https://software.broadinstitute.org/morpheus/) and hierarchical clustering was performed with this software using 1-Pearson's correlation unless otherwise stated. Correlation plots of samples were visualised using the similarity matrix tool from Morpheus.

De novo motif discovery

To analyse ATAC-seq or ChIP-seq peaks for enriched transcription factor motifs, genomic coordinates were analysed using findMotifsGenome.pl with –cpg –mask –size 200 -bg parameters from the Homer package (v4.7; Heinz et al., 2010). Background sequences were total accessible regions from all samples for ATAC-seq analysis and whole genome for ChIP-seq analysis.

Gene set enrichment analysis and gene ontology analysis

Pre-ranked genes (ranked by $\log_2$(fold change)) were subject to gene set enrichment analysis from hallmark gene sets (h.all.v6.2) using GSEAPreranked from GSEA v3.0 (Subramanian et al., 2005). Gene ontology analysis was carried out using Metascape (Zhou et al., 2019; metascape.org).

Footprinting analysis

To analyse footprinting signatures in ATAC-seq data the TOBIAS package was used (v0.5.1; Bentsen et al, 2019; available at https://github.molgen.mpg.de/loosolab/TOBIAS). Merged BAM files from each condition were processed using ATACorrect, footprint scores calculated using FootprintScores and differential footprinting analysis using BINDetect. Footprinting plots across identified footprints at TF motifs were plotted using plotProfile from the deepTools package (v2.5.0; Ramírez et al., 2016).

ChIP-seq and analysis

ChIP-seq was carried out as described previously (Wiseman et al., 2015). 1 x 10^7 cells, 5 µg target protein antibody, 1 µg Spike-in antibody (Active Motif, 61686) and 50 µl Protein A Dynabeads™ were used. 20 ng Spike-in *Drosophila* chromatin (Active Motif, 53083) was supplemented to chromatin preps for Spike-in normalisation, as described previously (Egan et al., 2016). Normal rabbit IgG (Millipore, 12-370) antibody was used in parallel as a control. DNA libraries were prepared using TruSeq ChIP sample prep kit (Illumina) and sequenced on a HiSeq 4000 (Illumina) platform. Sequencing reads were aligned to GRCh37 (hg19) and dm6 using Bowtie2 v2.2.3 (Langmead et al., 2009). Reads aligning to *Drosophila* genome were counted and used to generate scale factors. BAM files were then scaled to the sample with the lowest number of *Drosophila* reads. Only reads with a mapping quality >q30 were retained. Peak calling was performed on individual replicates using MACS2 v2.1.1 (Zhang et al., 2008) using default parameters with additional –SPMR parameter. bedGraph files were converted to bigwig using BedGraphtoBigWig script and visualised in the UCSC Genome Browser. The overlap of peaks between two biological replicates was calculated using BEDtools v2.26.0 (Quinlan and Hall, 2010) using bedtools intersect with default settings with -f 0.3 parameter. Peaks present in both datasets were taken forward for further analysis.

Differential binding analysis was performed using DESeq2 (Love et al., 2014). Overlaps from biological repeats were merged using bedtools merge to generate a final set of peaks. featureCounts from the SUBread package (Liao et al., 2014) was used to count reads within peaks from ChIP-seq samples and these were used an input for DESeq2 to calculate differential binding using default settings. A linear fold change of ±2 and a *Q*-value of <0.05 were used as a cut-off for further analyses.

ChIP-seq visualisation

Heatmaps of ChIP-seq signal were generated using computeMatrix and plotHeatmap from the deepTools package (Ramírez et al., 2016). Tag density plots were generated using computeMatrix and plotProfile tools from the deepTools package. Correlation of biological

replicates was visualised using multiBigwigSummary and plotCorrelation. Euler diagrams were generated using the Euler R package (available at eulerr.co).

Principal component analysis

Principal component analysis was performed using the prcomp function in R (v3.5.1, R Core Team, 2018) using $\log_2$ transformed RNA-seq or ATAC-seq normalised counts. Principal component scores were then plotted in Excel.

Patient survival analysis

Average expression of *KLF5* or KLF5 target genes (OAC upregulated and siKLF5 downregulated) was calculated per patient and patients were ranked by average target gene expression. The median was calculated and patients were classified as either above or under median expression. Survival (months) was plotted for each group and a Log-rank test was carried out using GraphPad Prism v8

Datasets

All data were obtained from ArrayExpress, unless stated otherwise. ATAC-seq data from human BO, OAC tissue and OE19 cells were obtained from E-MTAB-5169 (Britton et al., 2017) and E-MTAB-6751 (Rogerson et al., 2019). BO and OAC RNA-seq data were obtained from E-MTAB-4054 (Maag et al., 2017) and European Genome-phenome Archive (EGA)(EGAD00001005915). GATA6 and HNF4A ChIP-seq were obtained from E-MTAB-6858 and siGATA6 RNA-seq from E-MTAB-6756. The Cancer Genome Atlas OAC ATAC-seq data were obtained from the GDC data portal (portal.gdc.cancer.gov; Corces et al., 2018).

Data Access

All sequencing data are deposited in ArrayExpress. Additional OAC ATAC-seq data are available at E-MTAB-8447 and additional BO and OAC RNA-seq data are available at E-MTAB-8584. siKLF5 RNA-seq data are available at E-MTAB-8446. KLF5 ChIP-seq data are available at E-MTAB-8568. siERBB2 ATAC-seq and RNA-seq data are available at E-MTAB-8576 and E-MTAB-8579 respectively.

Acknowledgements

We thank Guanhua Yan for excellent technical assistance and staff in the Bioinformatics, and Genomic Technologies core facilities and Mike Jackson in the Flow Cytometry facility. We also thank Elizabeth Ratcliffe for collection of BO samples. We also thank Nicoletta Bobola and Shen-Hsi Yang for critical appraisal of the manuscript. This work was funded by grants from the Wellcome Trust (103857/Z/14/Z) and Cancer Research UK (through the Manchester Cancer Research Centre).

Author contributions: C.R., S.O. and EB performed the experiments and data analysis in this study; A.D.S. led the experimental parts of the program; and Y.S.A. led the clinical parts of the study. All authors contributed to manuscript preparation and/or critically appraised manuscript drafts.

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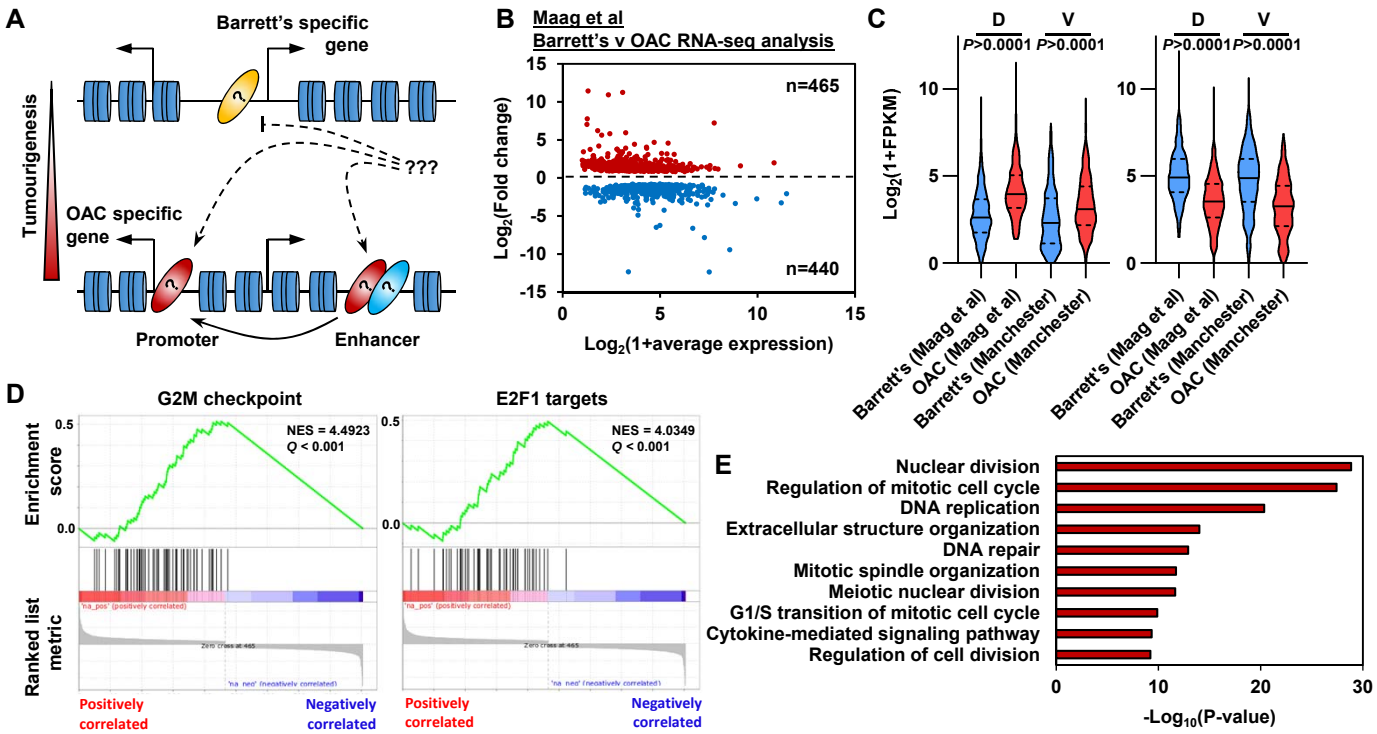

Figure 1. OAC tumourigenesis is associated with enhanced cell cycle gene activity. (A) Schematic of possible transcription factor network induction during OAC development. Coloured ovals represent different transcription factors. (B) Scatter plot of significant differentially (±1.5x, *Q*-value<0.05) expressed genes between human Barrett's oesophagus n=13 and human oesophageal adenocarcinoma n=12 samples (Maag et al., 2017). (C) Violin plots of expression of differentially expressed genes between Barrett's oesophagus (n=13) and oesophageal adenocarcinoma (n=12) from discovery dataset (D; Maag et al., 2017) and validation dataset (V; BO=3; OAC n=4). Genesets are shown for upregulated (left) and downregulated (right) in OAC. (D) Gene set enrichment analysis of differentially expressed genes. The top two upregulated gene sets are shown with normalised enrichment score (NES) and *Q*-value. (E) Biological pathway GO term analysis of upregulated genes. The top 10 terms are shown.

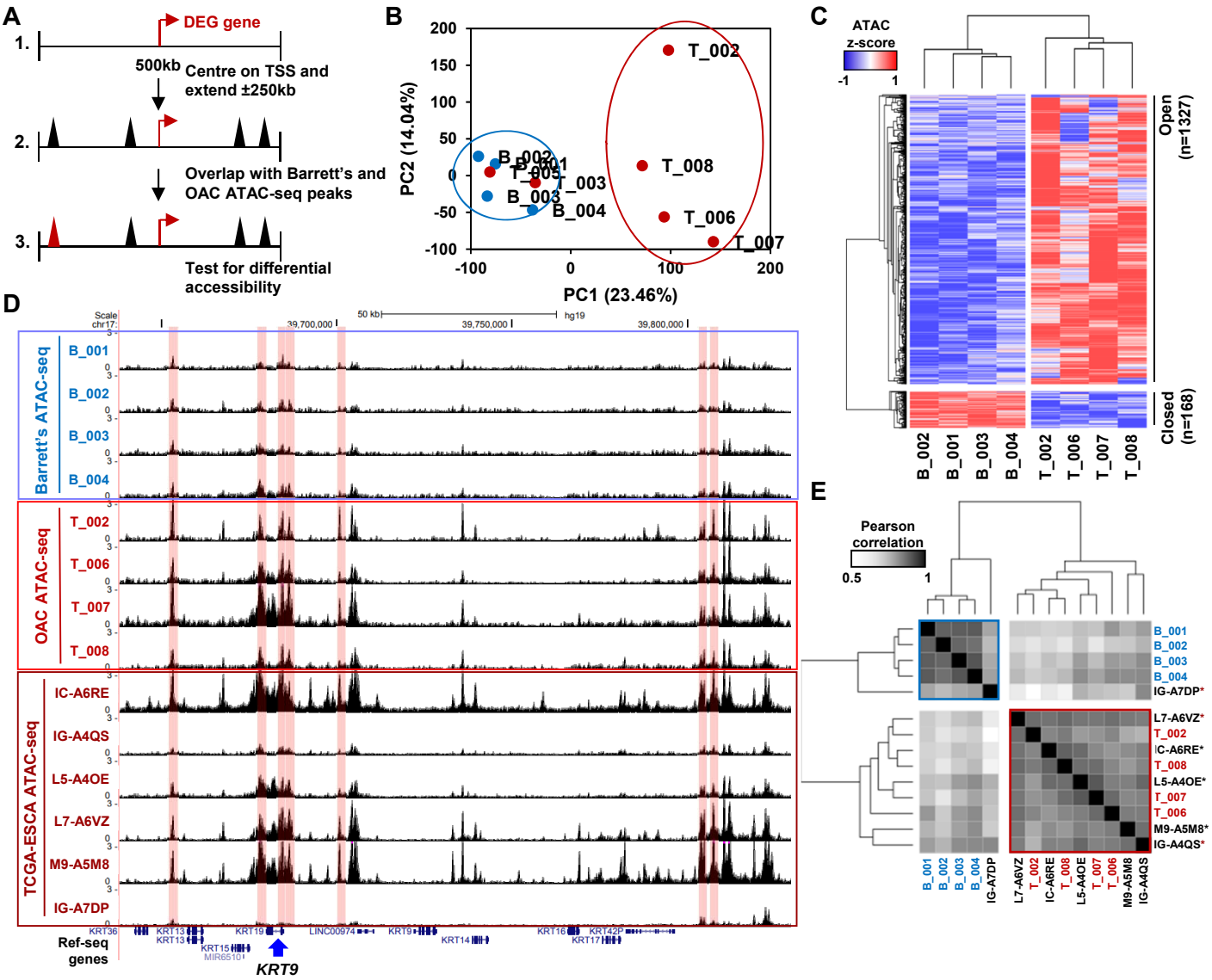

Figure 2. Altered chromatin accessibility landscape during OAC carcinogenesis. (A) Schematic of ATAC-seq analysis. All peaks within ± 250 kb of the TSS of a differentially expressed genes were assessed for differential accessibility between Barrett's oesophagus and OAC. (B) Principal Component Analysis plot of $\log_2$(1+FPKM) ATAC-seq signal from all accessible regions within ± 250 kb of a differentially expressed gene TSS from all human Barrett's oesophagus (B; n=4) and oesophageal adenocarcinoma samples (T; n=6). (C) Heatmap of *z*-score ATAC-seq signal from human Barrett's oesophagus (B; n=4) and OAC (T; n=4) samples at differentially accessible regions (± 2x; *Q*<0.1). Hierarchical clustering of samples and regions performed using 1-Pearson correlation. (D) Example UCSC browser view of BO, OAC and TCGA ESCA ATAC-seq data surrounding the *KRT19* locus with differentially accessible regions highlighted in red. (E) Correlation plot of Pearson correlation of $\log_2$(1+FPKM) ATAC-seq signal at differentially accessible regions. Hierarchical clustering performed using 1-Pearson correlation and the two main clusters are highlighted blue (BO) and red (OAC). TCGA samples are indicated by asterisks.

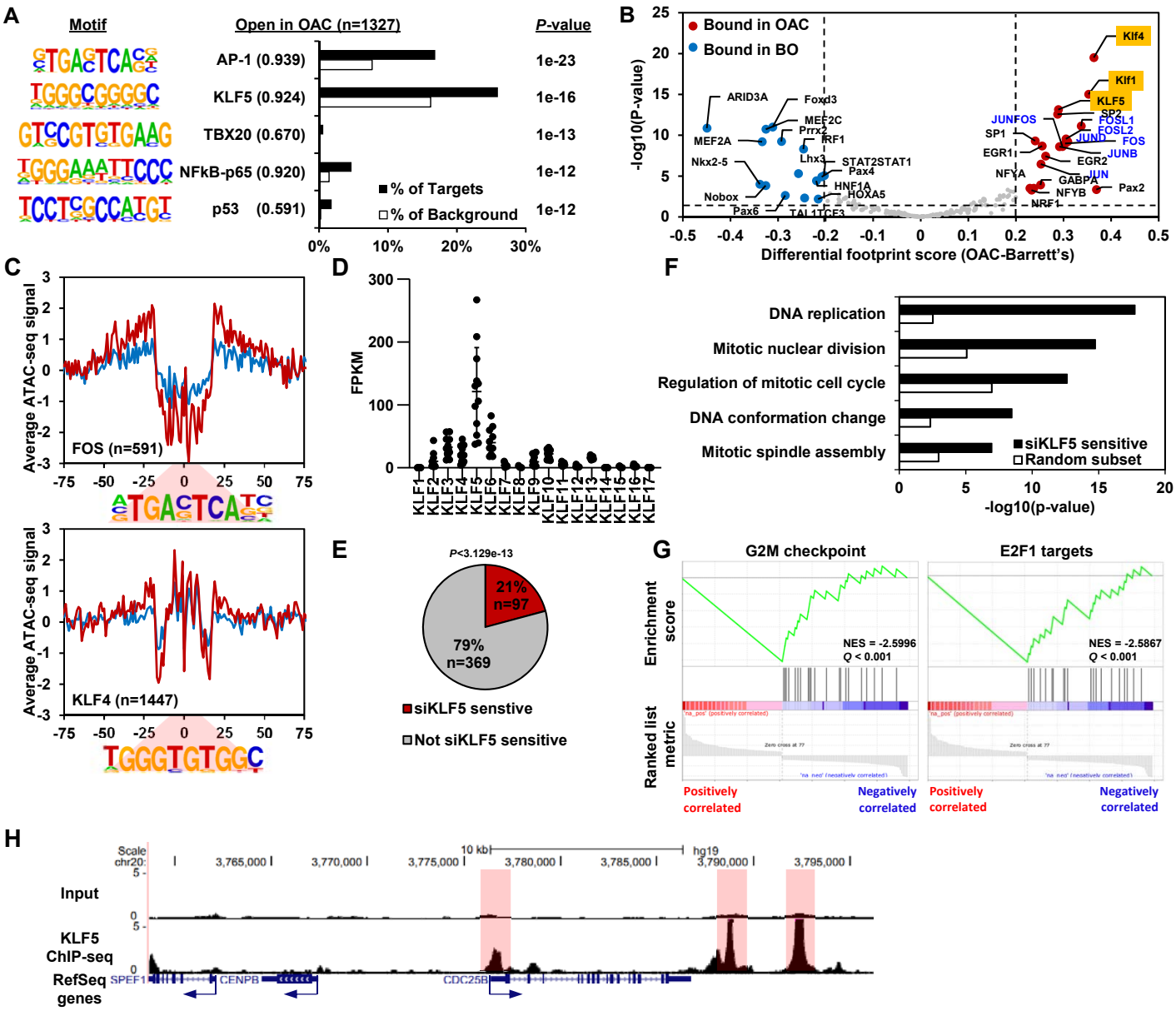

Figure 3. KLF5 control a cell cycle gene expression programme in OAC. (A) Bar chart of percentage targets and percentage background of de novo discovered motifs at increased accessible regions in OAC compared to Barrett's oesophagus. De novo motif, called transcription factor with motif match score (brackets) and *P*-value are shown. (B) Scatter plot of differential footprinting depth around human transcription factor motifs in differential accessible regions in BO and OAC tissue. Significant motifs with more footprint depth in BO are labelled blue and in OAC labelled red. KLF TF binding motifs are highlighted in orange and AP1 motifs are in blue font. (C) BO (blue) and OAC (red) ATAC-seq signal at FOS (AP1) and KLF4 (KLF) motifs in differentially accessible regions. (D) Expression (FPKM) of KLF family transcription factors in OAC RNA samples (n=12; Maag et al., 2017). (E) Pie chart of percentage of upregulated genes in patient OAC samples that are also downregulated with siKLF5 treatment. *P*-value shown. (F) Biological pathway GO term analysis of OAC upregulated and siKLF5 downregulated genes and a random gene selection. (G) Gene set enrichment analysis of genes that are upregulated in OAC and downregulated by siKLF5 treatment. Top two downregulated gene sets are shown. Normalised enrichment scores (NES) and *Q*-values are shown. (H) Example UCSC Genome Browser view of KLF5 ChIP-seq binding at the *CDC25B* locus. KLF5 peaks highlighted in red.

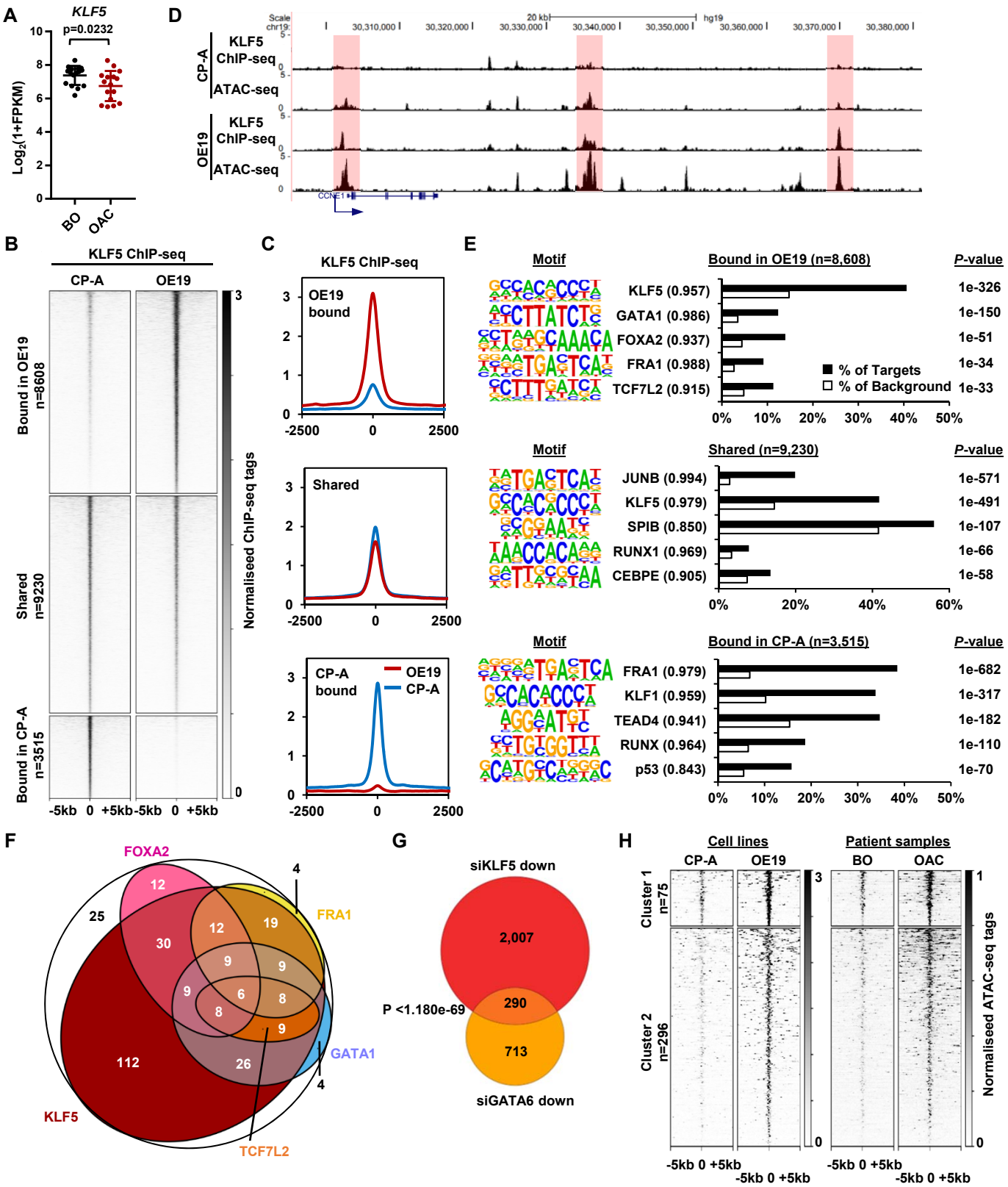

Figure 4. KLF5 binds to distinct regions in OE19 cells. (A) Expression (Log_2(1+FPKM) of *KLF5* in BO and OAC tissue. (B) Heatmap of KLF5 ChIP-seq signal at regions (peak centre ±5 kb) significantly bound in OE19 only (+2x; *Q*<0.05), shared regions (no significant change) and regions bound in CP-A only (-2x; *Q*-value<0.05). (C) Tag density plot of KLF5 ChIP-seq signal at regions (peak centre ±2.5 kb) bound in OE19 only, shared regions and regions bound in CP-A only. (D) Genome browser tracks showing KLF5 ChIP-seq and ATAC-seq in CP-A and OE19 cells at the *CCNE1* locus. Differential bound regions are highlighted in red. (E) Bar chart of percentage targets and percentage background of *de novo* discovered motifs at regions bound in OE19 only, shared regions and regions bound in CP-A only. *De novo* motifs, called transcription factor with match scores and *P*-values shown. (F) Euler diagram of 299 KLF5 binding regions that are specific to OE19-specific binding regions that are located within loci (+/-250 kb) containing genes upregulated in OAC and downregulated with KLF5 depletion. The motifs identified in Fig. 4E (KLF5, GATA1, FOXA2, FRA1 and TCF7L2) found within each peak are shown (note that an additional 71 regions cannot be depicted which contain combinations of motifs of the transcription factors other than KLF5). (G) Venn diagram showing the overlap in genes downregulated in OE19 cells following treatment with siRNAs targeting KLF5 and GATA6. (H) Heatmap of ATAC-seq signal at the KLF5 binding regions from (F) in the indicated cell lines (left) or patient derived tissue (right). Regions were subject to *k*-means hierarchical clustering (*k*=2).

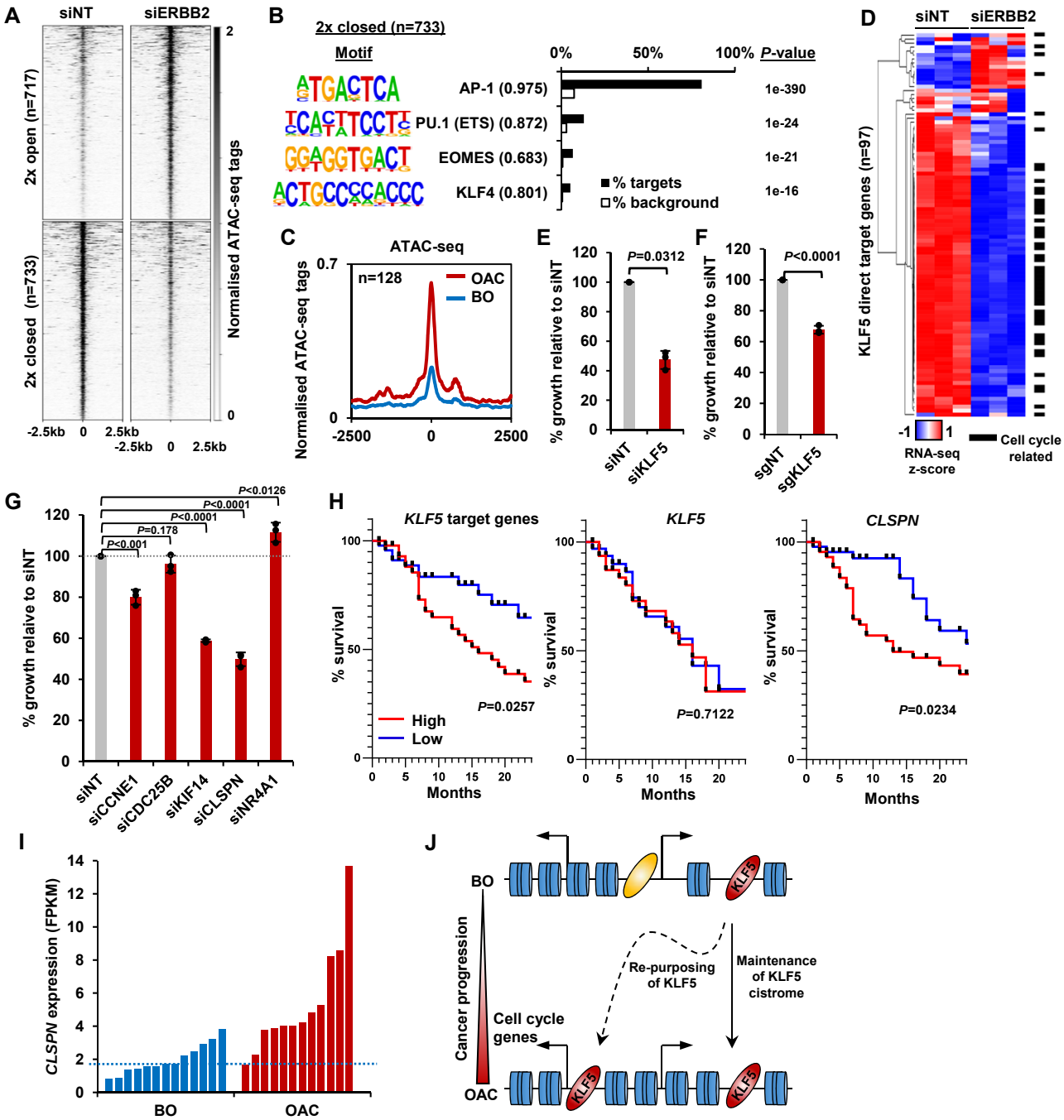

Figure 5. KLF5 controls cell proliferation in OAC. (A) Heatmap of ATAC-seq signal at regions (peak centre ±2.5 kb) with significantly differentially accessibility (±2x; Q<0.05) in OE19 cells treated with siERBB2. (B) Bar chart of percentage targets and percentage background of *de novo* discovered motifs at regions closed with siERBB2 treatment in OE19 cells. *De novo* motifs, called transcription factor with motif match scores (brackets) and P-values are shown. (C) Tag density plot of ATAC-seq signal from BO and OAC tissue at regions that demonstrate KLF5 binding in OE19 cells and reduced chromatin accessibility in OE19 cells upon siERBB2 treatment. (D) Heatmap of z-score of expression of KLF5 direct target genes in OE19 cells treated with either siNT or siERBB2. Cell cycle related genes are indicated with a black bar. (E) Bar chart showing the % relative growth of OE19 cells treated with either siNT or siKLF5. *P*-value is shown (n=3). (F) Bar chart showing the % relative growth of OE19-dCas9-KRAB cells treated with either non-targeting guides or guides targeting the KLF5 TSS. *P*-value is shown (n=3). (G) Bar chart showing the % relative growth of OE19 cells treated with either siNT or siRNA against the indicated target genes. *P*-values are shown (n=3). (H) Kaplan-Meier curves of patient survival across 24 months for high (above median; red) or low (below median; blue) expression of the 97 KLF5 target genes (left), *KLF5* (middle) or *CLSPN* (right). *P*-values are shown. (I) Expression levels (FPKM) of *CLSPN* expression in BO and OAC patient samples. (J) Model of KLF5 action in BO and OAC. KLF5 binds chromatin in BO and is re-purposed in OAC to bind and regulate cell cycle related genes.