TITLE

by Natasha Louise Hopkins

Master of Biology (Honours), Molecular Cell Biology
University of York, UK

Project Director
Prof. Robert J White

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Contents

TITLE

Natasha L. Hopkins

Abstract

1 Words

Introduction

tRNAs and Carcinogenesis

In Eukaryotes, transcription of DNA is tightly-regulated by three RNA polymerase enzymes. RNA Polymerase III (Pol III), the largest of the three at 17-subunits[1], produces a series of short non-coding RNAs, including U6 snRNA, 5S rRNA and transfer RNA (tRNA)[2]. Approximately 80% of Pol III binding resides at tRNAs[3] — adapters required to translate mRNAs into the amino acid protein sequence. Initiation of tRNA transcription requires the binding of TFIIIC, a multisubunit complex, to internal tRNA promoters, the A and B boxes, which are located downstream of the transcription start site[4–6]. This protein-protein interaction enables the recruitment of the TFIIIB promoter, composed of the TATA-box-binding protein (TBP), BDP1, and BRF1 polypeptides[7]. TFIIIB occupies the region upstream of the transcription start site and binds to Pol III directly through BRF[8], positioning it at the initiation region[9].

Protein synthesis by Pol III dictates cell growth and proliferation[10–12]. Deregulation of Pol II is associated with a range of cancers[13,14], including ovarian and breast[15,16]. In healthy cells, tumour suppressors such as RB and p52 regulate Pol III transcription. This is achieved through binding to TFIIIB, blocking interactions to both TFIIIC and Pol III[17–20]. Loss of RB and p53 in transformed cells consequently enhances Pol III transcription. Contrarily, induction of onco-proteins MAP kinase Erk or c-Myc may stimulate Pol II expression through interactions with TFIIIB[21,22]. The relationship between Pol III and cell transformation directly implicates tRNAs in carcinogenesis. ~~Tumour cells contain tRNAs that are absent from the normal tissue of origin[23].~~

Specific tRNAs have also been shown to drive cancer progression[24]. For instance, overexpression of the transcription initiator $tRNA_i^{Met}$ alters global tRNA expression, increasing cell activity and proliferation[25], as demonstrated in melanoma cells[26]. ~~Reporter assays revealed that some tRNA genes (tDNAs) act as insulators by restricting spread of heterochromatin[27–29].~~

ER+ Breast Cancer and FOXA1

In 2020, female breast was the most commonly diagnosed cancer worldwide with over 2.2 million cases[30]. Estrogen Receptor (ER) drives 75% of breast cancers cancers[31]. Presence of ER is generally prognostic of positive outcomes, due to it's use as a therapeutic target. Estrogen levels are lowered using

endocrine therapies with aromatase inhibitors; fulvestrant, a selective estrogen receptor down regulator (SERD) which binds and degrades the ER, and tamoxifen, a selective estrogen receptor modulator (SERM) which competes with estrogen for binding to ER[32]. However, resistance to endocrine therapy occurs in 40-50% of tumours from 5 years of diagnosis[33].

Forkhead box A1 (FOXA1) is a pioneer factor capable of directly initiating chromatin opening, facilliating binding of ER and other transcription factors[34–37]. Early studies demonstrated that FOXA1 maps to ˜50% of ER-chromatin binding sites. Futhermore, silencing of FOXA1 reduces ˜95% ER binding events[36,38].

~~FOXA1 is an established growth inhibitor in both ER+ breast cancer cells and ER- cells with forced ER expression[39]. Though binding to $p27^{kip1}$, FOXA1 is also involved in recruiting tumour suppressor BRCA1[40–42].~~

The presence of FOXA1 determines treatment response in ER+ breast cancer; FOXA1 is associated with positive prognoses following treatment with tamoxifen[38,43]. However, FOXA1 and ER has also been found to be overexpressed in some ER+ endocrine resistant breast cancer lines[44]. Studies have shown that amplification of FOXA1 promotes endocrine-resistant growth and invasiveness by reprogramming the ER-transcriptome[45].

Aberrant expression of tRNAs in breast cancers has been observed in several studies[13,14,16,46]. Upon stimulation by estrogen, ER+ breast cancer cells undergo extensive transcriptional changes, upregulating 90% of tRNA genes[46]. Additionally, ER amplifies alcohol-induced deregulation of $tRNA^{Leu}$ by in MCF-7 cells[47].

As ER+ BC is reliant on FOXA1, the aim of this study was to determine whether FOXA1 elevates tDNA expression. To achieve this, a bioinformatics approach was taken to analyse publically available FOXA1 and H3K27ac ChIP-seq datasets from Fu et al. (2019)[48] in the context of the MCF-7 cell line. Here, identification of FOXA1 and H3K72ac co-localisation at tDNAs may provide insight into FOXA1's relevance in the altered tRNA expression associated with poor prognosis in ER+ breast cancer.

Materials & Methods

ChIP-seq Data from NCBI

ChIP-seq was performed on genetically modified MCF7L cells (*insertion, using a lentiviral cDNA delivery system to express Dox-inducible FOXA1*) by the lab of Xiaoyong Fu, Baylor College of Medicine, and made publicly available on Dec 18 2019.[48]. Datasets were deposited into the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) Run Selector under the accession number PRJNA513000 (Available at https://www.ncbi.nlm.nih.gov/sra ; Table 1). Using "Genetic Manipulation Tools" within the Galaxy[49] environment (v 23.0.rc1), SRAs were converted to FastQ files. FastQ files were then aligned to the human genome assembly GRCh37 (hg19) using Bowtie2 (v 2.5.0)[50] to output BAM files.

Table 1. Publicly available ChIP-seq SRA files aquired from the NCBI SRA database (accession no. PRJNA512997).

Experiment	SRA	Factor	Tissue	Assembly
PRJNA513000	SRR8393424	FOXA1	MCF-7LP	GRCh37 (Hg19)
	SRR8393425			
	SRR8393426			
	SRR8393427	H3K27ac		
	SRR8393428			
	SRR8393431	None (input)		
	SRR8393432			

EaSeq for the Quantification of Signals at tDNAs

BAM files were uploaded into EaSeq (v1.111) as "Datasets" using the standard settings for Chip-seq data. GRCh37 (hg19) tRNA sequences (n = 606) were downloaded as a "Geneset" from the UCSC Table Browser[51], (available at https://genome.ucsc.edu). High-confidence tRNAs (n = 416) identified by the GtRNAdb[52] were extracted as a "Regionset".

Signal peak intensities surrounding tRNAs were quantified using the EaSeq "quantify" tool. Here the default settings "Normalize to reads per million" and "Normalize counts to DNA-fragments" were left checked. The default setting "Normalise to a signal of 1000 bp" was unchecked. The window size was offset ±500bp from the start of each tRNA gene. Outputs are referred to as "Q-values". To quantify upstream and downstream signals, the "quantify" tool was used with adjusted window sizes. The upstream region was defined as 500 bp preceding and the first nucleotide of tRNA loci. Thus, the start position was offset to 0 bp, and the end position was offset to -500 bp. The downstream region constitutes the 500 bp region beginning with the first nucleotide of tRNA gene body. The start position was offset to 1 bp, and the end position was offset to 500 bp. Following quantification, tRNA binding events were arranged in ascending order -DOX Q-value and visualised as heatmaps. Data was also visualised with "average", and "overlay" EaSeq tools.

EaSeq[53] is avaiable at http://easeq.net.

Motif Analysis

Motif analysis was carried out in active +Dox tDNA genes which had the largest significant increase in FOXA1 or H3K27ac Q-values following ChIP-seq.

!!1!!!!1 !!!!!!1

Multiple EM for Motif Elicitation ChIP (MEME) Suite

Statistics

Statistical analysis and visualisation was generated with R[54] (v 4.2.3). Significant difference was determined by Mann-Whitney U, Wilcoxon signed-rank test, and the Chi-squared test.

ChIP-Seq and ChIP-qPCR

MCF-7L cells were grown in PRF medium with 5% CS-FBS and /+ Dox.Cells were cross-linked with 1% formaldehyde for 10 mins. Corss-linking was inhibited by quenching (1/20V, 125 mM glycine). Cells were washed in cold PBS and harvested in cold PBS with protease inhibitors (Roche). Pelleted cells were resuspended in cytosolic and then lysed in nuclear lysis buffer (10-20 min), and sheared at high output (Bioruptor, Diagenode; 4 ˚C, 30s per sonication cycle for 20 min). Sonicated lysates were cleared by centrifugation (20,000 × g, 10 min) and diluted (4 times) before preincubation with protein-A/G beads (Santa Cruz; 4 ˚C, 30 min).

ChIP was performed by overnight incubation (4 ˚C) with antibody against human FOXA1 (Abcam, ab23738) or H3K27ac (Active Motif, #39134), followed by an additional incubation with protein-A/G beads (1h). For FOXA1 ChIP-seq, spike-in Drosophila melanogaster chromatin was added with the antibody against histone variant H2Av (Active Motif, # 61752). The beads were washed with low and high salt wash buffer, once with LiCl wash buffer (20 mM Tris pH 8.0, 1 mM EDTA, 250 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate), and once with TE buffer. DNA was eluted (50 mM NaHCO3 and 1% SDS) and then supplemented with NaCl (300 mM). Cross-links were reversed by incubating overnight (67 ˚C). RNA was digested at 37 ˚C with RNase A (0.1 mg/mL, 30 min). DNA was purified with a PCR purification kit (Qiagen).

Indexed libraries were prepared from ChIP DNA using the KAPA Hyper Library Preparation Kit (Kapa Biosystems). Libraries were amplified by PCR (12 cycles), and then assessed for size distribution using the 4200 TapeStation High Sensitivity D1000 ScreenTape (Agilent Technologies) and quantified using the Qubit dsDNA HS Assay Kit (ThermoFisher). The indexed libraries were multiplexed, 10 libraries per pool. Real time quantitative PCR (qPCR) was perfomed using the KAPA Library Quantification Kit (KAPA Biosystems) and then sequenced on the Illumina NextSeq500 using the high-output 75 bp single-read configuration.

Results

Localisation of FOXA1 and H3k27ac to tRNA Genes

Mapped reads of FOXA1 and H3K27ac binding were visualised as heatmaps and ordered by increasing -DOX Q-value. This revealed a concentration of FOXA1 and H3k27ac at approximately half of tRNAs, relative to ±10 kb flanking regions. FOXA1 OE increased FOXA1 binding at a small proportion of tRNAs genes, and H3K27ac binding decreases at approximately half of tRNA genes (Figure 1A). This was confirmed by average signal intensity plots of FOXA1 and H3K27ac binding (Figure 1B). Input reads generated minimal peak enrichment (Supplementary Figure X).

Peaks were classified as binding events if Q-values exceeded input values (Figure 1C). FOXA1 interacts with 329 tRNA genes and H3K27ac with 293 tRNA genes. FOXA1 co-binds with 89.4% of H3K27ac sites. Upon FOXA1 OE, 40 FOXA1 binding sites are lost and 30 are gained. H3K27ac sites are lost at 50 tDNAs, and 23 are gained. Here, FOXA1 co-binding represents 92.5% of H3K27ac sites.

Upon FOXA1 OE, mean Q-values significantly increased 1.18-fold for FOXA1 binding ($p < 0.0001$), and significantly decreased 0.86-fold for H3K27ac ($p < 0.01$) (Supplementary Figures X). However, FOXA1 OE leads to a significant difference in FOXA1 and H3K27ac binding between individual tDNAs ($p < 0.0001$) (Figure 1D).

Together, these results support the notion that FOXA1 overexpression alters the binding landscape of FOXA1 and H3K27ac at tRNAs.

Figure 2

Why?

How does FOXA1 alter H3K27ac binding?

Using Q-values, tRNAs that are differently enriched upon FOXA1 OE were categorised as 'UP' or 'DN' (FOXA1 = 359, H3K27ac = 315).

This discovered substantially more tRNAs with increased (UP) than decreased (DN) FOXA1 (92 vs. 21) (Figure 2A). However, for H3K27ac, the number of tRNAs with an increase (UP) was comparable to those with a decrease (DN) (41 vs. 44) (Figure 2B).

#REWRITE %s

Of the tDNAs which gain (UP) H3k27ac, 51% (21) also gain (UP) FOXA1; none lose (DN) FOXA1.

Of the tDNAs which lose (DN) H3k27ac, 22.7% (10) also lose FOXA1, with just 1 tDNA gaining FOXA1 (Figure 2C).

Examples of these tRNAs are shown in Figure 2D.

Figure 3

The next step was to investigate the impact of FOXA1 on the number of active genes. Almost half of human tDNAs are silent or poorly expressed[55]. Thus, tRNAs were classified as 'active' if H3K27ac Q-values exceeded the median -DOX value ($Q > 1.808$).

When FOXA1 is over-expressed, the activity status of the majority (87.3%) of genes remain unchanged. The number of of tDNAs that lose and gain activity are 27 and 26, respectively.

Of the +Dox activated genes, 12 also gained FOXA1.

FOXA1 alone is insufficient in increasing tRNA activity.

Suggests?

Figure ?

- Relative position (not very interesting)
- Binding at isotopes? Certain AA more up than others?

Figure 4

Why?

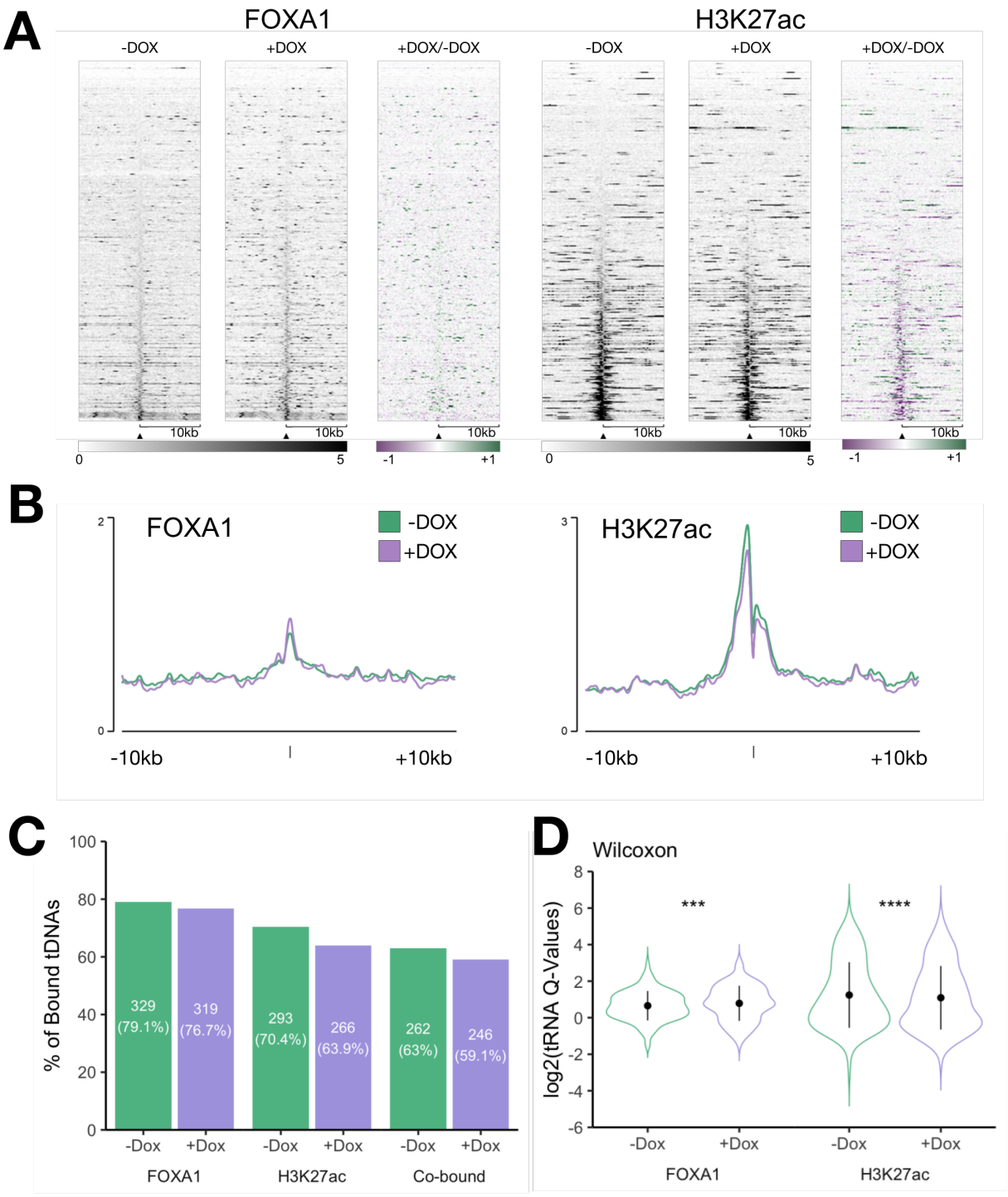

Figure 1. Comparisons of FOXA1 and H3K27ac binding at tDNAs, with and without Dox.(A) Heatmaps of FOXA1 and H3K27ac across hg19 tRNA genes in MCF-7 cells. Genes arranged in order of increasing -Dox Q-value. Ratiometric heatmaps represent the log2 ratio between -Dox and +Dox peaks. Windows represent ±10kb from the centre of the gene.(B) Average signal intensity overlay of FOXA1 and H3K27ac. Windows represent ±10kb from the centre of the gene. (C) Bar plots of the percentage of cells bound by FOXA1 and H3K27ac. (D) Violin plots of FOXA1 and H3K27ac Q-values within ±500bp of the gene body. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$.

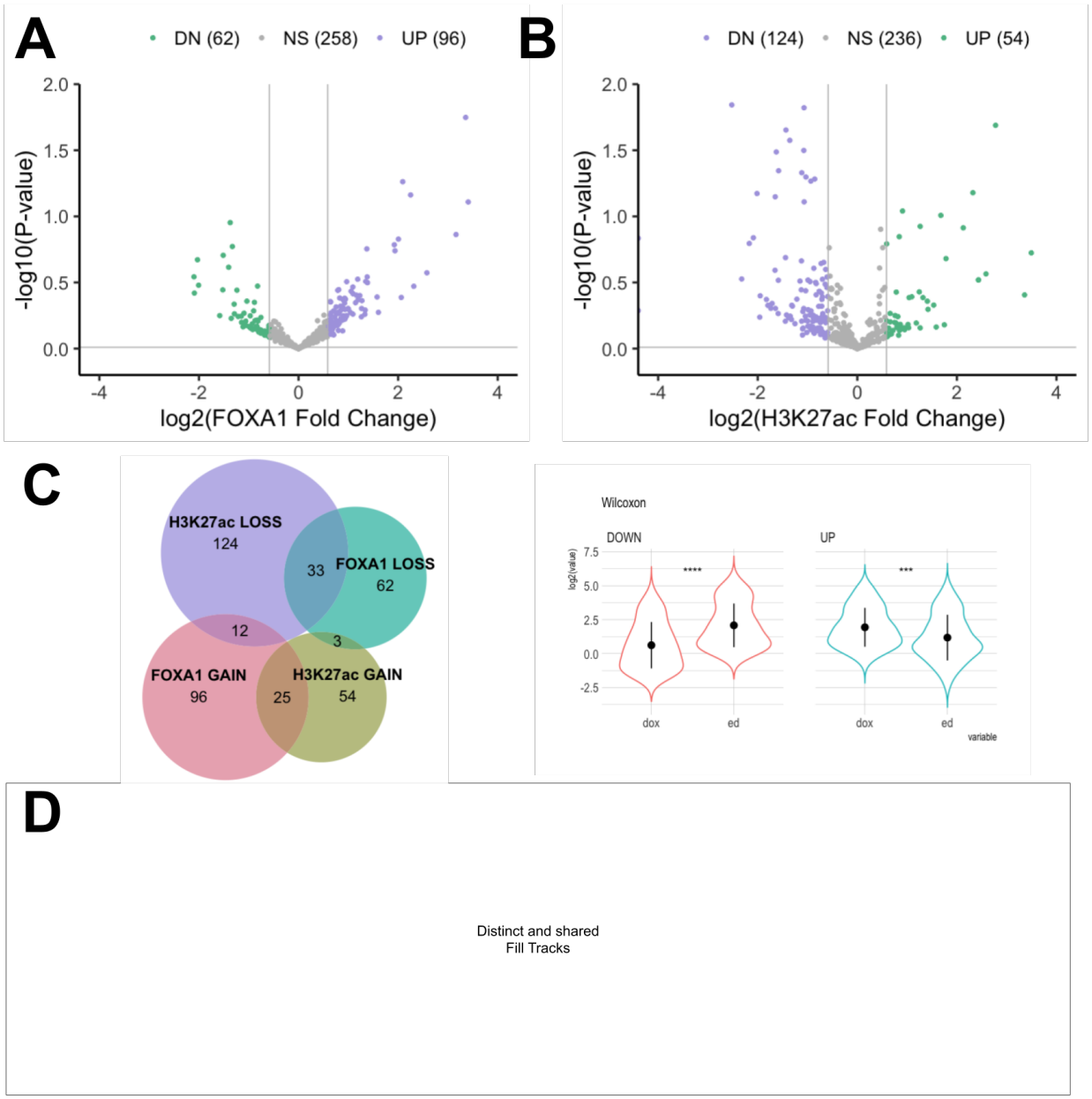

Figure 2. FOXA1 OE impacts binding of FOXA1 and H3K27ac at tDNAs. (A and B) Volcano plots of FOXA1 (A) and H3K27ac (B) Q-values with and without Dox. The purple and green dots correspond to the regions with UP and DN expression in +Dox vs. Dox cells, respectively. (C) Venn diagram representing the overlap between Dox-induced FOXA1 and H3K27ac UP and DN regions. (D). (E)

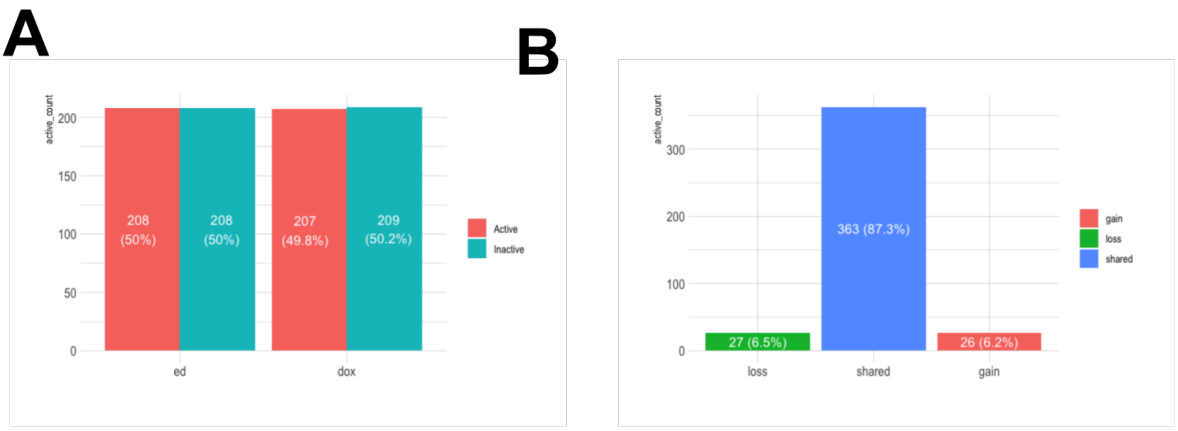
A
B
active_count
200
150
100
50
0
208
(50%)
208
(50%)
207
(49.8%)
209
(50.2%)
ed
dox
Active
Inactive
active_count
300
200
100
0
27 (6.5%)
363 (87.3%)
26 (6.2%)
loss
shared
gain
gain
loss
shared

Figure 3. .

What?

MEME CentriMo to identify de novo motifs that are enriched at tRNAs which gain both FOXA1 and H3k27ac

- relative to other tRNAS (gain/lose, lose/lose, lose/gain)
- FOXA1 not enriched
- Look at ERE, AP-1, others?
- Top 3 motifs (fisher E values) - IRF7, ERR3, NR4A1
- A and B box motifs as a control? How?
    - All downstream
    - tRNAs where 'matching sequences' all best matches = code for valine
    - Branched amino acids associated with lower BC risk

Suggests?

Figure 5

- Localisation of FOXA1 at individual tRNA genes in MCF-7 cells

Why?

- tRNAs implicated in cancer
- Are they upregulated?
- Look at gain function/gain h3/fox
- Motif ontology
-

What?
Suggests?

Table 2. .

Group	Function
ALOXE	Insulator Function[27,29]
Ebersole	Insulator Function[28,29]
HES7	
Per1	

Group	Function
TMEM107	Insulator Function[27,29]
Arg-CCG	Implicated in Cancer Progression[24]
Glu-TTC	Implicated in Cancer Progression[24]
iMET	Proliferation of Breast Cancer
Met	iMet Control
SeC	Involved in REDOX[56]

Discussion

- FOXA1 alone not efficient to increase activity

  { p300

- FOXA1 moves nucleosomes to make other TF accessible?
- Differences in nucleosome positioning will contribute to the distinct distribution patterns of modified histones, as nucleosomes are excluded from active tRNA promoters and enriched in flanking regions[8].
- Brf1 required for iMET txn
- Loses fox = weak binding?
- Dynamic vs stable marks
- ATAC-seq

Conclusion

2153 Words

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Venn of Motif inputs

De novo Identified Motifs

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