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Gene expression analysis

Expression data-sets

Two gene expression data-sets were generated, a total B cell (totalB) and a negatively selected (CD23 depletion) memory B cell set (memB; referred to as m. in figures), both from 3 healthy donors, from day-0 (resting B-cell; totalB only) on day-3 after activation with CD40L/anti-BCR/cytokines (ABC) and then at intervals of 3h, 6h, 12h, 24h, 48h and 72h (PB) after transition into conditions (continued IL2 and IL21 only) that support the ABC/PB transition. A G9A inhibited data-set (G9A_i) was generated for the totalB data with the

inclusion of the small molecule inhibitor UNC0638 at day-3, with samples taken at the same intervals as the standard conditions.

Normalisation and analysis

Illumina GenomeStudio Gene Expression Module was employed for initial data processing followed by the R Lumi package (v2.36.0).¹ Probes not detected on three or more arrays were removed and the remaining data variance-stabilizing transformed (VST) and quantile normalized. A linear model was fitted to the gene expression data using the R Limma package (v3.40.6).² Differentially expressed genes between the Standard/G9A_i contrasts were gauged using the Limma empirical Bayes statistics module, adjusting for multiple testing using Benjamini and Hochberg correction. Probes were re-annotated using the MyGene.info (<http://mygene.info>) API using all available references (e.g. NCBI Entrez, Ensembl etc.) and any ambiguous mappings manually assigned.³ Finally, probes for each gene were merged by taking the median value for probe sets with a Pearson correlation ≥ 0.2 and the maximum value for those with a correlation < 0.2 (as used by Monti *et al*).⁴

PGCNA networks

Background

For details and validation of the Parsimonious Gene Correlation Network Analysis (PGCNA) approach see our other work.⁵ Here a brief description of the method will be given. After informative genes are selected they are used to calculate Spearman's rank correlations for all gene pairs using the Python `scipy.stats` package. For each gene (row) in a correlation matrix only the 3 most correlated edges per gene are retained. The resulting matrix M , with entries written as $M = (m_{ij})$ is made symmetrical by setting $m_{ij} = m_{ji}$ for all indices i and j so that $M = M^T$ (its transpose). The correlation matrices are clustered using a community detection algorithm (Louvain v0.3 or Leidenalg v0.7.0; see PGCNA2) and the 100 best (judged by modularity score) used for downstream analysis.^{6,7}

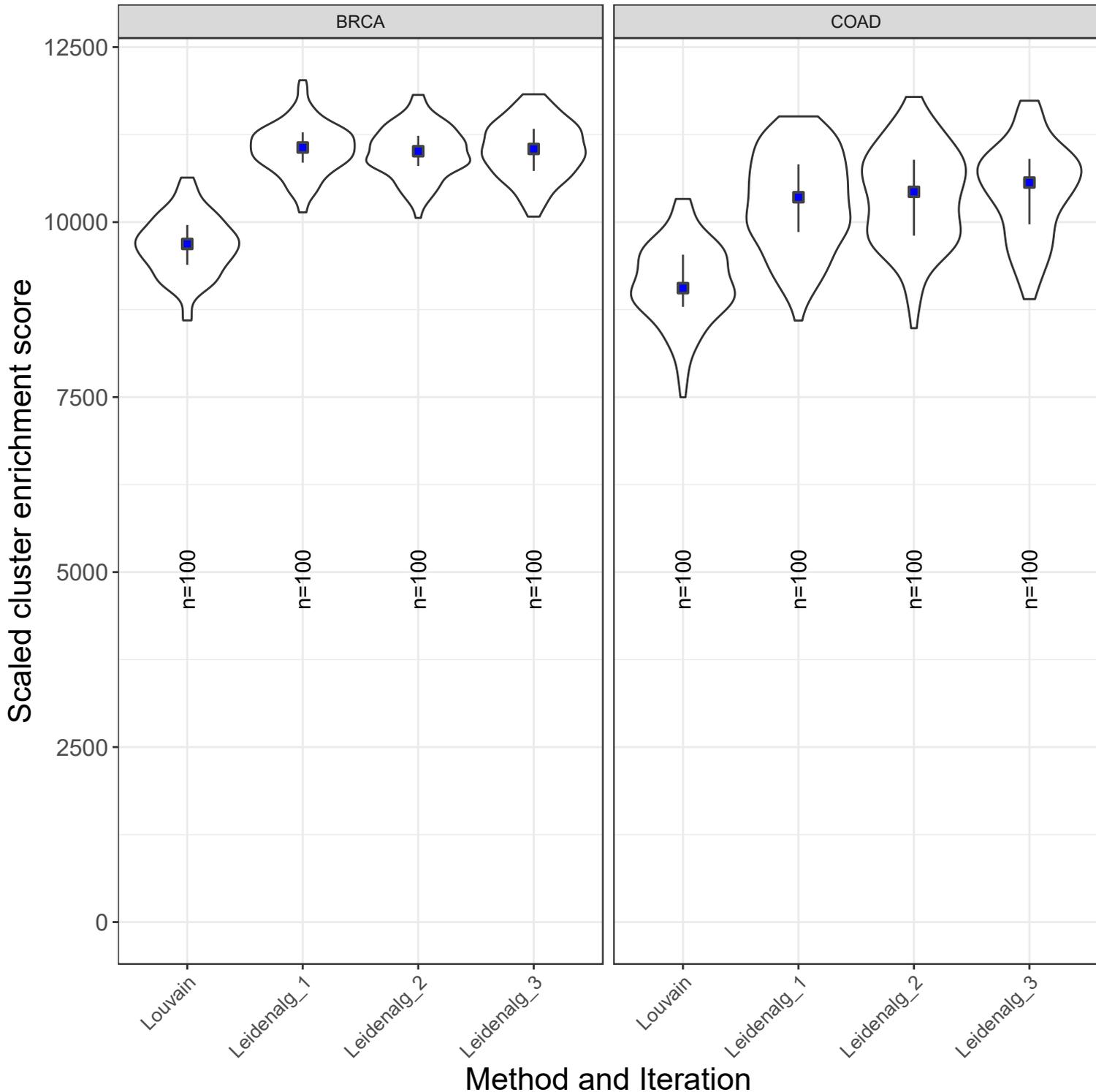
Data preparation

The totalB and memB standard condition time course data were used for PGCNA network construction. Before probes were merged per-gene the most informative probes were selected for the different time courses. All probes with a $\sigma^2 > 0.025$ (across median values per time point) were selected, giving 11,418 and 10,183 probes for the totalB and memB sets respectively. The selected probes were used to filter the complete (not merged across donors) data-sets and finally the probes merged per gene. This gave 9,362 and 8,431 genes for the totalB and memB data-sets.

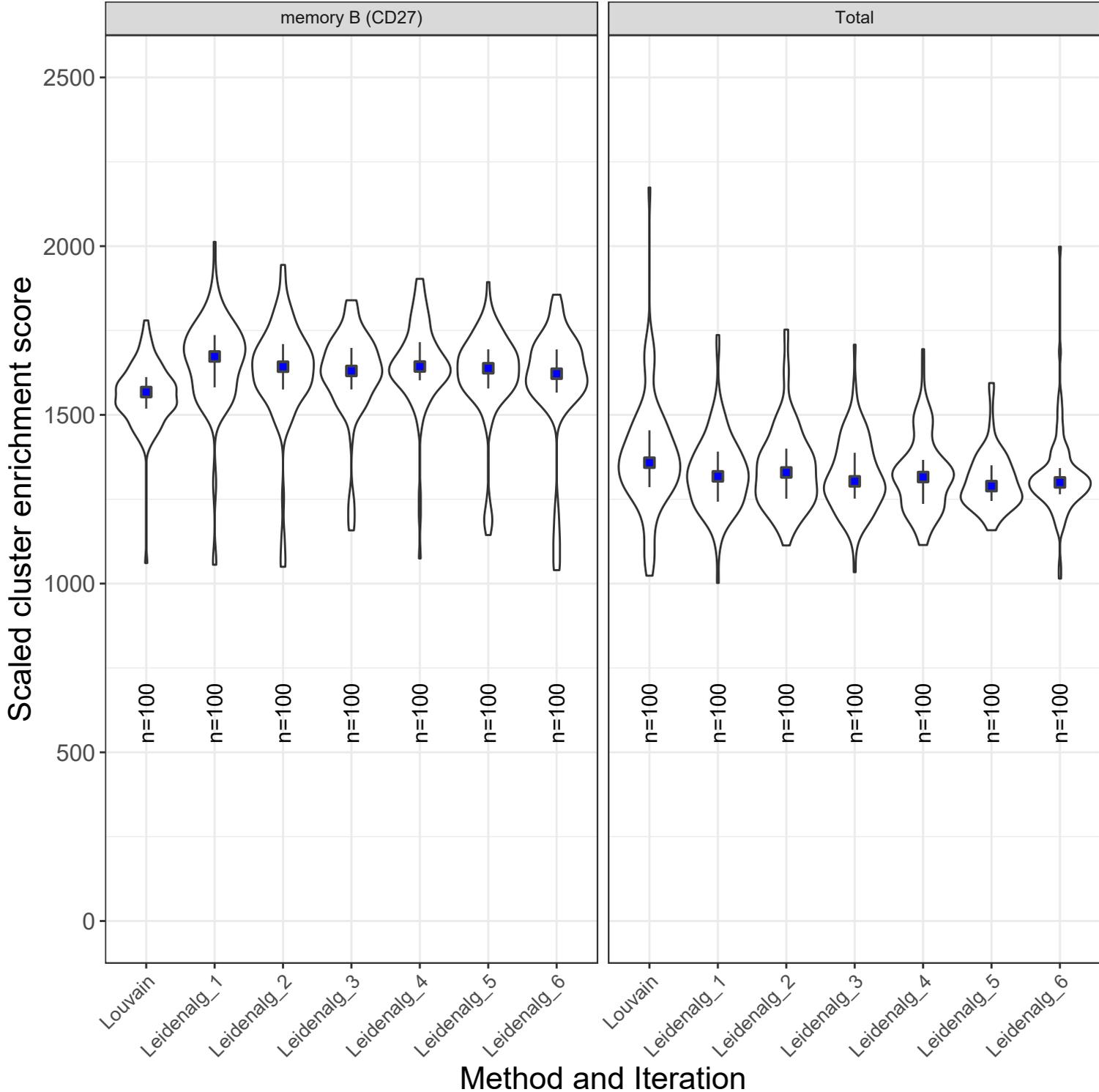
PGCNA2 network generation

For this project the community detection method used by PGCNA (Louvain/FastUnfold) was replaced with an improved algorithm that guarantees well-connected communities (Leidenalg).^{6,7} In order to test if the Leidenalg improved the results of the PGCNA approach it was compared to the Louvain method for the BRCA/COAD data-sets (used in the original PGCNA paper) and the totalB and memB data-sets (used here) using a Spearman correlation matrix reduced to 3 edges per gene (EPG).⁸ For the BRCA/COAD analysis the

Louvain results were compared to 3 rounds of Leidenalg runs, whilst for the smaller totalB/memB data-sets the Louvain results were compared to 6 rounds of Leidenalg runs. For the Louvain method each round consisted of 10,000 iterations, whilst for Leidenalg each round consisted of 1,000 iterations (with optimiser set to iterate until no partition improvement). For both Louvain/Leidenalg for each round the top 100 results (judged by modularity score) were then analysed for biological enrichment against a signature database (see Gene signature data) by generating Scaled cluster enrichment scores (see original PGCNA methods for details).⁵ Methods Figure 1 and 2 show that in 3 of the 4 tested data-sets Leidenalg improved the biological enrichment. The optimal Leidenalg results were then used for downstream visualisation and analysis. This resulted in two networks: PGCNA-totalB (9,362 genes, 22,921 edges) and PGCNA-memB (8,431 genes, 20,524 edges).



Methods Fig 1. BRCA/COAD. Enrichment of gene ontology and signatures was assessed using a scaled cluster enrichment score (SCES) and compared between data clustered with Louvain (FastUnfold) or Leidenalg for parsimonious matrices with edges per gene (EPG) of 3. Violin plots display the distribution along with median (blue square) and the IQR.



Methods Fig 2. memory/Total. Enrichment of gene ontology and signatures was assessed using a scaled cluster enrichment score (SCES) and compared between data clustered with Louvain (FastUnfold) or Leidenalg for parsimonious matrices with edges per gene (EPG) of 3. Violin plots display the distribution along with median (blue square) and the IQR.

ChIP-seq analysis

Data-sets

File	TF	Condition	TotalReads	TotalAligned	%Aligned
H929_1_IRF4	IRF4	Cell-Line	32,217,322	27,426,682	85.13
H929_1_Input	-	Cell-Line	31,547,003	24,791,189	78.58
U266_1_IRF4	IRF4	Cell-Line	35,388,343	19,744,836	55.79
U266_1_Input	-	Cell-Line	33,047,885	26,231,974	79.38
OCILY3_1_IRF4	IRF4	Cell-Line	34,319,995	26,245,026	76.47
OCILY3_1_Input	-	Cell-Line	35,482,443	31,100,345	87.65
OCILY3_2_IRF4	IRF4	Cell-Line	22,112,257	18,172,619	82.18
OCILY3_2_Input	-	Cell-Line	11,652,330	7,346,745	63.05
OCILY10_1_IRF4	IRF4	Cell-Line	30,282,459	19,520,680	64.46
OCILY10_1_Input	-	Cell-Line	33,739,395	28,515,619	84.52
OCILY10_2_IRF4	IRF4	Cell-Line	41,055,313	35,335,492	86.07
OCILY10_2_Input	-	Cell-Line	16,179,020	13,205,999	81.62
Day3BC_1_IRF4_Std	IRF4	Std	14,402,873	11,928,556	82.82
Day3BC_1_Input_Std	-	Std	76,638,168	55,935,640	72.99
Day3BC_2_IRF4_Std	IRF4	Std	14,465,135	12,004,663	82.99
Day3BC_2_Input_Std	-	Std	76,830,340	59,330,110	77.22
Day6PB-B_XBP1_Std	XBP1	Std	26,511,959	21,722,657	81.94
Day6PB-B_Input_Std	-	Std	29,074,912	23,855,970	82.05
Day6PB-memB_BLIMP1_Std	BLIMP1	Std	35,002,987	29,416,625	84.04
Day6PB-memB_CTCF_Std	CTCF	Std	36,105,839	31,556,061	87.40
Day6PB-memB_H3K27Ac_Std	H3K27ac	Std	54,433,687	47,458,206	87.19
Day6PB-memB_H3K4me3_Std	H3K4me3	Std	53,132,933	43,282,575	81.46
Day6PB-memB_IRF4_Std	IRF4	Std	34,346,524	28,691,545	83.54
Day6PB-memB_Input_Std	-	Std	26,944,620	14,681,441	54.49
Day6PB-memB_BLIMP1_0638	BLIMP1	UNC0638	26,740,354	22,690,984	84.86
Day6PB-memB_H3K27Ac_0638	H3K27ac	UNC0638	39,826,235	34,929,002	87.70
Day6PB-memB_H3K4me3_0638	H3K4me3	UNC0638	40,544,784	33,485,297	82.59
Day6PB-memB_Input_0638	-	UNC0638	21,062,154	11,530,118	54.74

Alignment and peak discovery

Before aligning, reads were trimmed to remove adapters and low confidence regions using a python script. A 4 base sliding window was run along each read, if the average Q Phred score for a window was < 20 the read was trimmed at the window start, finally any match to adapter sequences were trimmed.

Trimmed reads were aligned to GRCh38 (UCSC analysis set) using Bowtie2 (v2.3.5.1) with the `--very-sensitive` parameter.⁹ The resultant SAM files were converted to BAM using samtools (v1.9) with the quality filter set to 20 (`-q 20`).¹⁰ The BAM files were analysed for peaks using GEM (v3.4), with quality filter set to 1 (`-q 1`) and MACS2 (v2.1.2) with quality filter set to 0.05 (`-q 0.05`).^{11,12}

The resultant BAM files were converted to BED files and read cross-correlation was assessed using MaSC (v1.2.1).¹³ Reads were extended to the estimated fragment length, and a scaled (reads per million; rpm) BED file generated. This was converted to a coverage file using the UCSC genomeCoverageBed tool and then to a BigWig file using UCSC bedGraphToBigWig for peak visualisation.¹⁴

Peak overlap and filtering

ChIP-seq peak overlaps were used in two contexts. Firstly, to reduce false positive peak discovery a high-confidence merged peak set was created by overlapping the GEM/MACS2 peaks. Secondly, the resultant high-confidence peaks were used to find the overlap between the different transcription factors (TFs) creating a non-redundant set of binding regions. The peak centres for all data-sets were ordered per chromosome. Starting at the beginning of each chromosome peaks were added to a cluster. New peaks were only added to the cluster if the distance between their centre and the cluster's centre was ≤ 250 bp, else a new cluster was started. As new clusters were generated if peaks in the earlier cluster were closer to the new cluster's centre they were moved into the new cluster (thus a peak can only belong to one cluster). The minimum GEM/MACS2 $-\log_{10}Q$ for a peak to start a new cluster was set to ≥ 5 , however, this was lowered to 1 for the addition of peaks to existing clusters. Note that the number of peaks in the TF overlap sets are less than the individual peaks per TF due to the merging of some close peaks into a single peak cluster.

For the cell-line IRF4 data, first a high-confidence set was created per sample by overlapping the GEM/MACS2 peaks. For the multiple myeloma (H929/U266) cell-lines the two high-confidence sets were overlapped, while for the DLBCL (OCILY3/OCILY10) cell-lines the 4 high-confidence sets were overlapped.

For the day 3 IRF4 samples, the GEM/MACS2 peak sets were combined per run and then a high confidence set created by finding their overlap, generating a set of peak clusters that have at least one GEM/MACS2 peak in both runs.

This gave the final individual high-confidence sets: BLIMP1 (n=4,323), D3.IRF4 (n=18,271), D6.IRF4 (n=9,512) and XBP1 (n=605) along with the Union set consisting of all peaks in the overlap of BLIMP1_D6.IRF4_XBP1 (n=12,491) and a Union.2 set consisting of all peaks in the overlap of BLIMP1_D3.IRF4_D6.IRF4_XBP1 (n=27,649).

The full results from peak merging and overlap analysis can be found in Supplemental Table 5.

Peak annotation

Version 28 of the Gencode gene annotation data was downloaded from UCSC, the genes were re-annotated using the HUGO Gene Nomenclature Committee annotations (2018/06/08 version).^{14,15} Each peak was assigned the nearest gene (TSS) as its primary gene. In addition annotatePeaks.pl from the HOMER suite (v4.11.1) was used to provide detailed peak annotations (Promoter: -1kb – 100bp, TTS: -100bp – 1kb, Exonic/Intronic: > 100bp from Promoter/TTS within gene, Intergenic: >1kb from Promoter/TTS outside gene).¹⁶

Motif discovery

BED files were generated for the peak overlap sets, ± 125 bases around each peak centre (or overlap cluster centre). These were analysed for *de novo* motifs of length 8 – 14 using findMotifsGenome.pl from the HOMER suite.

Data visualisation

Network visualisation

The optimal PGCNA-totalB/PGCNA-memB networks were converted to a list of edges and nodes and uploaded into the Gephi package (version 0.9.2).¹⁷ Degree and Betweenness Centrality were calculated, and the latter used to adjust node sizes. The network layout was generated using the ForceAtlas2 approach, and interactive HTML5 web visualizations exported using the sigma.js library (<https://github.com/oxfordinternetinstitute/gephi-plugins/tree/sigmaexporter-plugin>). The interactive visualisations can be found at <https://mcare.link/ABCtoPB>.

Heatmaps

The gene expression data and GSE results were both visualised using the Broad GENE-E package (<https://software.broadinstitute.org/GENE-E/>). For visualisation of expression data, the timepoint median expression values were row-normalised (z-scores), while for GSE visualisation the signature enrichment/depletion z-scores were used. In both cases the data was hierarchically clustered (Pearson correlations and average linkage).

ChIP-seq clustering and visualisation

The high-confidence peak sets for BLIMP1, IRF4 and XBP1 along with the Union/Union.2 set (overlap of individual high-confidence BLIMP1, D3.IRF4, D6.IRF4 and XBP1 peaks; see supplemental Table 5) were analysed using the deepTools2 suite (v3.3.0).¹⁸ Using bamCoverage peaks were normalised to bins per million mapped reads (BPM) and extended to their MaSC estimated fragment length (e.g. --normalizeUsing BPM --extendReads 140 --binSize 10). Scores per region were calculated with computeMatrix using a BED file reference for a ± 1000 bp region (--referencePoint center -b 1000 -a 1000 --skipZeros). The resulting matrix was k-means clustered and then visualised using plotHeatmap (--kmeans 6).

Graphs

All graph visualisations were generated using the R package ggplot2 (v3.2.1), with the viridis (v0.5.1) colour-blind friendly colour scheme.¹⁹

Venn diagrams

Venn diagrams were generated using the R package VennDiagram (v1.6.20).

Motif logos

The *de novo* motifs generated by HOMER were converted to information scaled pdfs using the python API for the WebLogo package (v3.6.0).²⁰

Statistical analyses

Gene signature data

A data-set of 17,904 gene signatures was created by merging signatures downloaded from <http://lymphochip.nih.gov/signaturedb/> (SignatureDB), <http://www.broadinstitute.org/gsea/msigdb/index.jsp> MSigDB V6.2 (MSigDB C1–C7 and H; excluding C5. With MIPS signatures from version 3.1 and PID signatures from version 4 added back), <http://compbio.dfci.harvard.edu/genesigdb/> Gene Signature Database V4 (GeneSigDB), UniProt keywords (parsed XML from <http://www.uniprot.org/downloads>), and fifteen papers.^{4,21,30–37,22–29} A gene ontology gene set was created using an in-house python script. This parses a gene association file (<http://geneontology.org/page/download-go-annotations>) to link genes with ontology terms and then uses the ontology structure (.obo file; <http://purl.obolibrary.org/obo/go.obo>) to propagate these terms up to the root. The resultant gene set contained 22,782 terms. The gene-ontology and gene-signatures sets were merged to give a final signature set of 40,686 terms.

Enrichment analysis

The gene signature enrichment (GSE) was assessed using a hypergeometric test, in which the draw is the gene list genes, the successes are the signature genes, and the population is the genes present on the platform. The resultant p-values are then adjusted for multiple testing using Benjamini and Hochberg correction. GSE was carried out in three different contexts: to assess the biological enrichment of the PGCNA-totalB/PGCNA-memB network modules, to compare the TF bound genes against the PGCNA-memB network and finally to compare the G9A_i differentially expressed genes against the PGCNA-memB network.

For the PGCNA network GSE analyses the genes per module were compared against the 40,686-signature database (background: all the genes in that network). For analysis of TF bound genes against the PGCNA-memB modules the peaks were assigned to the nearest TSS (≤ 10 kb), the resultant genes were then compared against the genes per PGCNA-memB module (background: PGCNA-memB genes). Finally, for the G9A_i enrichment assessment the 72 hours differentially expressed genes (FDR < 0.05, FC > 1.2) were compared against the genes per PGCNA-memB module (background: PGCNA-memB genes).

Data processing

All analyses were undertaken on MARC1, part of the High Performance Computing and Leeds Institute for Data Analytics (LIDA) facilities at the University of Leeds, UK

Data and software availability

Interactive networks and all meta-data are available at <https://mcare.link/ABCtoPB>. PGCNA python scripts are available at <https://github.com/medmaca/PGCNA>. All ChIP-seq and expression data are available at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE142494>.

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