Supplementary Information

RTNsurvival case studies: regulon activity as a predictor variable in univariate and multivariate survival analyses.

Clarice S. Groeneveld[1], Vinícius S. Chagas[1], Steven J. M. Jones[2], A. Gordon Robertson[2], Bruce A. J. Ponder[3], Kerstin B. Meyer[3,4], Mauro A. A. Castro[1,*].

[1]Bioinformatics and Systems Biology Lab, Federal University of Paraná, Curitiba, 81520-260, Brazil. [2]Canada's Michael Smith Genome Sciences Center, BC Cancer Agency, Vancouver, V5Z 4S6, Canada. [3]Department of Oncology and Cancer Research UK Cambridge Institute, University of Cambridge, Li Ka Shing Centre, Cambridge, United Kingdom. [4]Wellcome Sanger Institute, Hinxton, CB10 1SA, United Kingdom.

*Corresponding author: mauro.castro@ufpr.br

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1. METABRIC breast cancer cohort 1

1.1 Context

For the METABRIC breast cancer cohort, Castro *et al.* (2016) described a survival analysis that used regulon activity to sort samples in the cohort, which was then stratified and evaluated by Kaplan Meyer (KM) and Cox regression approaches. The authors also described 36 transcription factors (TFs) that were associated with genetic risk of breast cancer. For these 36 TFs, Fletcher *et al.* (2013) reconstructed regulons using the cohort's microarray transcriptome data (Curtis *et al.*, 2012). Our goals in this section are, for the METABRIC cohort 1 (n=997): (1) to estimate regulon activity for these 36 TFs in individual samples, (2) to use regulon activity to sort and stratify the samples, considering sorted covariates, and (3) to assess regulon activity as predictor variable in univariate and multivariate survival analyses.

1.2 Package installation and data sets

The *RTNsurvival* package is available from the R/Bioconductor repository, together with other required packages. Installing and then loading the *Fletcher2013b* data package will make available all data required for this case study.

```r
#-- Set the Bioconductor repository
#-- Please make sure to use bioc version >= 3.8 (R >= 3.5)
source("https://bioconductor.org/biocLite.R")
biocVersion()
```

```r
#-- Install RTNsurvival and other required packages
#-- RTNsurvival (>=1.4.4); Fletcher2013b (>=1.16.0); RTN (>= 2.6.2)
biocLite(c("RTNsurvival","Fletcher2013b"))
install.packages("pheatmap")
```

```r
#-- Call packages
library(RTNsurvival)
library(Fletcher2013b)
library(pheatmap)
```

```r
#-- Load 'rtni1st' data object, which includes regulons and expression profiles
data("rtni1st")
```

The `rtni1st` data also provides clinical and molecular information for 997 samples from the METABRIC cohort 1 (Curtis *et al.*, 2012). The following variables are included in the `rtni1st` data: time to disease-specific death (*time*), event death (*event*), age (*Age*), tumour grade (*Grade*, *G1*, *G2* and *G3*), tumour size (*Size*), lymph nodes (*LN*), ER status from IHC (*ER+* and *ER-*), PAM50 subtypes (*LumA*, *LumB*, *Basal*, *Her2*, and *Normal*), hormone therapy (*HT*) and ethnicity (*Ethnicity*).

```r
#-- Check available attributes in 'colAnnotation'
colAnnotation <- tni.get(rtni1st, what="colAnnotation")
head(colAnnotation)
```

```r
#-- A list of transcription factors of interest (here, 36 risk-associated TFs)
risk.tfs <- c("AFF3", "AR", "ARNT2", "BRD8", "CBFB", "CEBPB", "E2F2", "E2F3", "ENO1",
              "ESR1", "FOSL1", "FOXA1", "GATA3", "GATAD2A", "LZTFL1", "MTA2", "MYB",
              "MZF1", "NFIB", "PPARD", "RARA", "RB1", "RUNX3", "SNAPC2", "SOX10",
              "SPDEF", "TBX19", "TCEAL1", "TRIM29", "XBP1", "YBX1", "YPEL3", "ZNF24",
              "ZNF434", "ZNF552", "ZNF587")
```

1.3 Data preprocessing

The data preprocessing consists of a single step that creates a TNS-class object. This step uses the tni2tnsPreprocess function, which requires (1) a transcriptional regulatory network computed by the *RTN* package, and (2) a list of regulators.

```
#-- Create TNS-class object from the 'rtni1st'
tns1st <- tni2tnsPreprocess(tni = rtni1st, regulatoryElements = risk.tfs,
                            time = "time", event = "event", endpoint = 120,
                            keycovar = c("Age","Grade"))
```

1.4 Regulon activity of individual samples

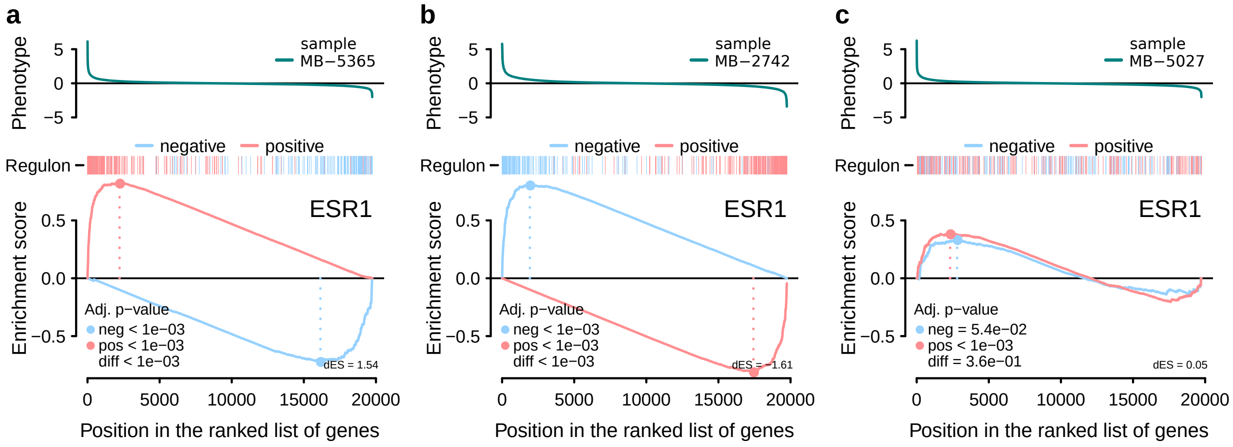
The tnsPlotGSEA2 function estimates a regulon activity score for a single sample in a cohort, using a two-tailed Gene Set Enrichment Analysis (GSEA-2T). In GSEA-2T, a regulon's positive and negative targets are each considered separate as *pos* and *neg* gene sets. These gene sets are evaluated against a differential gene expression signature, which differs between samples, and is typically calculated in *RTNsurvival* as follows: For each gene in a sample, a differential gene expression is calculated from its expression in the sample relative to its average expression in the cohort; the genes are then ordered as a ranked list representing a differential gene expression signature, also called the sample's phenotype. **Supplementary Figure 1a** shows the estimation of ESR1 regulon activity for a single tumour sample from the METABRIC breast cancer cohort. For each gene set (*pos* and *neg*) a walk down the ranked list is performed, stepwise. When a gene in the gene set is found, its position is marked in the rug plot, with the colour corresponding to the gene set. A running sum, shown as the pink and blue (*pos* and *neg* gene sets, respectively) lines, increases when the gene at that position belongs to the gene set and decreases when it doesn't. The maximum distance of each running sum from the x-axis represents the enrichment score. GSEA-2T produces two per-sample enrichment scores (ES), whose difference (dES = ES_{pos} - ES_{neg}) represents the regulon activity. The goal is to assess, for each sample, whether the target genes are overrepresented among the genes that are more positively or negatively differentially expressed. For a sample within a cohort, a large positive dES indicates an *induced (activated)* regulon, while a large negative dES indicates a *repressed* regulon. Luminal A sample MB-5365 has an activated pattern for ESR1 (**Supplementary Figure 1a**), while basal-like sample MB-2742 has a repressed pattern (**Supplementary Figure 1b**). The regulon status is assigned as *undetermined* when ES_{pos} and ES_{neg} distributions are skewed to the same side of the ranked list of genes (**Supplementary Figure 1c**).

```
#-- Two-tailed GSEA plots for individual samples
tnsPlotGSEA2(tns1st, "MB-5365", regs = "ESR1")
tnsPlotGSEA2(tns1st, "MB-2742", regs = "ESR1")
tnsPlotGSEA2(tns1st, "MB-5027", regs = "ESR1")
```

1.5 Regulon activity profiles

Regulon activity profiles (RAPs) seek to characterize regulatory program similarities and differences between samples in a cohort. In order to assess a large number of samples, we implemented a function that computes the two-tailed GSEA for the entire cohort. For each regulon, the tnsGSEA2 function estimates a regulon activity score for each sample in the METABRIC cohort 1.

```
#-- Compute regulon activity for individual samples (this may take a while)
#-- ...for a faster (parallel) option, please see the 'tnsGSEA2' documentation
tns1st <- tnsGSEA2(tns1st)
```



Supplementary Figure 1: Example of using a two-tailed GSEA to calculate ESR1 regulon activity in individual tumour samples. The *phenotype* is the sample's differential gene expression signature, which is obtained by comparing the expression of each gene in the current sample with its average expression across all samples in the cohort. The *phenotype* is used to generate the ranked list of genes on which the two-tailed GSEA is carried out for positive and negative targets (red and blue bars, respectively). For sample PAM50 LumA MB-5365 (**a**) the ESR1 regulon is activated (dES>0), while for sample PAM50 basal-like MB-2742 (**b**) the ESR1 regulon is repressed (dES<0). Sample MB-5027 (**c**) represents an inconclusive case, with positive and negative targets skewed to the same side of the ranked list of genes. These plots reproduce results from Castro *et al.* (2016).

Supplementary Figure 2 shows a heatmap of regulon activity profiles across the METABRIC cohort, together with tumour ER+/- status and PAM50 subtypes. To a large extent, regulon activity segregates samples into meaningful tumour subtypes. These results are consistent with previous studies showing that regulon activity can be used to sort samples in a cohort (for details, examples and additional interpretations on using the dES metric, please refer to Campbell *et al.* (2016), Castro *et al.* (2016), Robertson *et al.* (2017) and Campbell *et al.* (2018)).

```
#-- Get regulon activity and sample attributes
regact_gsea <- tnsGet(tns1st, "regulonActivity")$dif
sdata <- tnsGet(tns1st, "survivalData")
attribs <- c("ER+", "ER-","LumA","LumB","Basal","Her2","Normal")
```

```
#-- Plot regulon activity profiles
pheatmap(t(regact_gsea), annotation_col = sdata[,attribs], show_colnames = FALSE,
         annotation_legend = FALSE, clustering_method = "ward.D2",
         clustering_distance_rows = "correlation",
         clustering_distance_cols = "correlation")
```

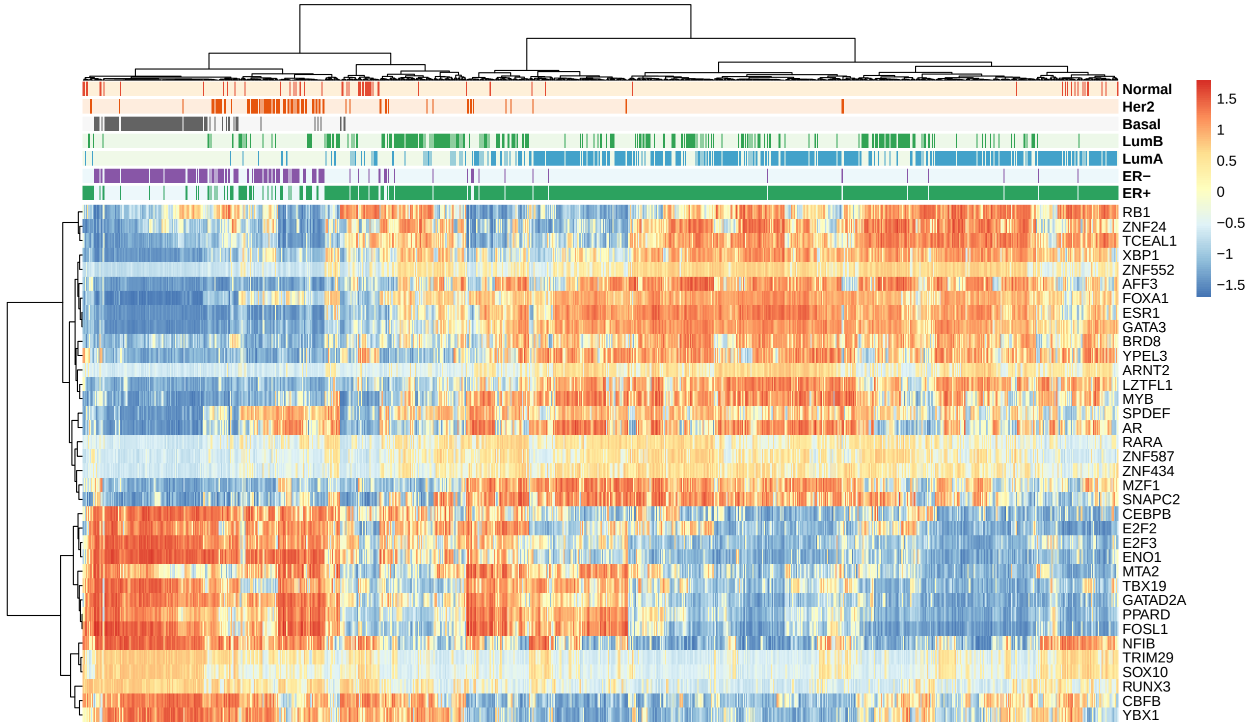
1.6 Univariate and multivariate survival analyses with RTNsurvival

The *RTNsurvival* package uses regulon activity as a predictor variable to study associations between regulons and survival. The `tnsKM` function can be used to generate Kaplan-Meier curves for one covariate (*i.e.* regulon) at a time. **Supplementary Figure 3a** separates the METABRIC cohort (n=997 samples) into three strata according to ESR1 regulon activity (dES<0, undetermined, and dES>0), and **Supplementary Figure 3b** shows the corresponding Kaplan-Meier curves. High ESR1 regulon activity is strongly associated with better survival (log-rank P = 1.96e-08), reproducing results from Castro *et al.* (2016). **Supplementary Figures 3c-d** illustrate an inverse case, with high PPARD regulon activity associated with poorer survival (log-rank P = 1.03e-07). This representation is very convenient for describing the predictor variable along with sample attributes (covariates) and survival curves.

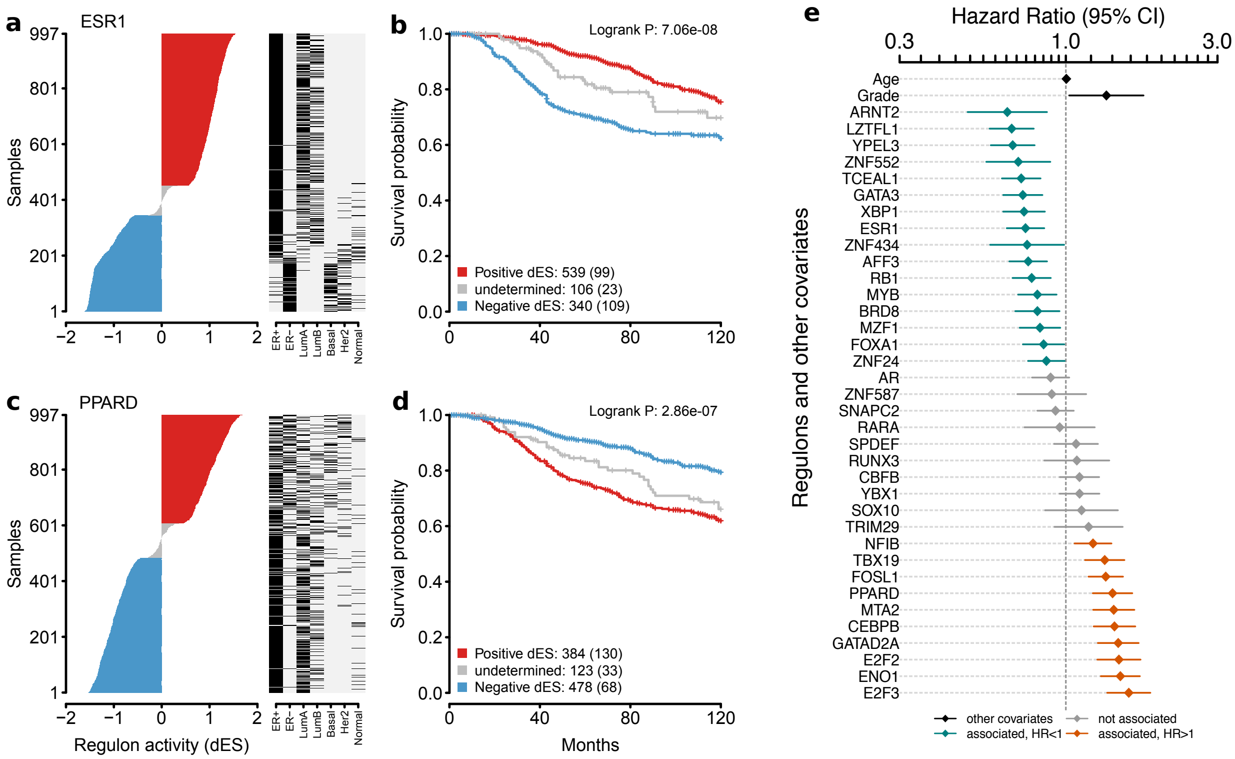
```
#-- Run KM analysis for regulons
tns1st <- tnsKM(tns1st)
tnsPlotKM(tns1st, regs = "ESR1", attribs = attribs, panelWidths=c(3,1,4), width = 6)
tnsPlotKM(tns1st, regs = "PPARD", attribs = attribs, panelWidths=c(3,1,4), width = 6)
```

Additionally, in order to study the main effects of survival predictors in a multivariate analysis we use the `tnsCox` function, which can adjust the analysis by including confounding factors or other covariates. This function relates the activity of one regulon to times-to-events in a multivariate, additive Cox proportional hazards model, and generates a graphic showing the calculated hazard ratios (HR). **Supplementary Figure 3e** shows that within the 36 regulons there are two subsets with statistically significant hazard ratios (HR $<$ 1 or HR $>$ 1, 95% CI). The regulons associated with with higher risk have higher activity values in ER- tumours, particularly basal-like tumors; conversely, regulons associated with lower risk have higher activity in ER+ tumours (**Supplementary Figure 2**).

```
#-- Run Cox analysis for regulons
tns1st <- tnsCox(tns1st)
tnsPlotCox(tns1st, height = 7)
```



Supplementary Figure 2: Unsupervised hierarchical clustering of regulon activity profiles across the 997 samples of METABRIC cohort 1 for the set of 36 TFs associated with genetic risk of breast cancer described in Castro *et al.* (2016).



Supplementary Figure 3: Univariate and multivariate survival analyses for single regulons. For the ESR1 regulon: (**a**) Left: stratification by ESR1 regulon activity (dES) of all 997 samples in METABRIC cohort 1. Samples with inconclusive regulon activity (*i.e.* undetermined status) are indicated in grey. Right: ER status and PAM50 subtypes. (**b**) Kaplan-Meier survival curves for the dES groups highlighted in (a). Numbers indicate patients in each group and, in curved parentheses, deceased patients (results reproduced from Castro *et al.*, 2016). (**c-d**) As in (a,b), for the PPARD regulon. (**e**) Cox multivariate analysis for 36 risk-associated regulons, each considered with age, grade, and regulon activity, for disease-specific survival in the METABRIC cohort 1.

1.7 Identification of proliferation-related regulons

Previous literature has indicated challenges in gene set-based survival analysis. Shimoni (2018) described a “random bias” that was attributed to a large proliferation signature that affects a substantial proportion of the genes in the genome. The author implemented a method that removes the bias by adjusting the gene expression data. The method is largely based on the meta-PCNA signature described by Venet *et al.* (2011), which consists of 131 genes that are associated with proliferation in breast cancer. Shimoni (2018) used the meta-PCNA signature to adjust gene expression for a large number of other cancer types. We used the meta-PCNA signature in our original study (Castro *et al.*, 2016) to identify regulons associated with proliferation in breast cancer, following a method that we described in Fletcher *et al.* (2013). The method consists of an enrichment analysis where we test which regulons are enriched with the meta-PCNA genes. Since the meta-PCNA signature was inferred in breast cancer, we can apply it to the METABRIC cohort.

In this example we show how to identify regulons enriched with the meta-PCNA signature. From our 36 risk TFs, only 3 regulons (E2F2, E2F3 and ENO1) are enriched with the signature. All three are linked to poor outcomes, consistent with their enrichment with proliferation markers. Please refer to Castro *et al.* (2016) and Fletcher *et al.* (2013) for additional details.

```
#-- Load meta-PCNA signature available from Fletcher2013b data package
data("miscellaneous")
```

```
#-- Run MRA analysis pipeline
rtna1st <- tni2tna.preprocess(rtni1st, hits=metaPCNA)
rtna1st <- tna.mra(rtna1st)

#-- Check regulons enriched with meta-PCNA genes
metaPCNA_enriched <- tna.get(rtna1st, what="mra")
```

Table 1: Top 10 regulons enriched with meta-PCNA signature

Regulon	Pvalue	Adjusted.Pvalue
PTTG1	1.0e-49	5.7e-47
FOXM1	1.9e-34	5.5e-32
E2F2	6.1e-24	1.1e-21
E2F8	2.1e-23	2.9e-21
HMGB2	1.7e-16	1.9e-14
ILF2	5.3e-13	5.0e-11
VENTX	5.3e-11	4.3e-09
ZNF395	9.1e-11	6.5e-09
TGIF2	1.1e-10	6.6e-09
PURA	7.0e-10	4.0e-08

```
intersect(metaPCNA_enriched$Regulon, risk.tfs)
```

```
## [1] "E2F2" "E2F3" "ENO1"
```

1.8 Other metrics for assessing regulator activity

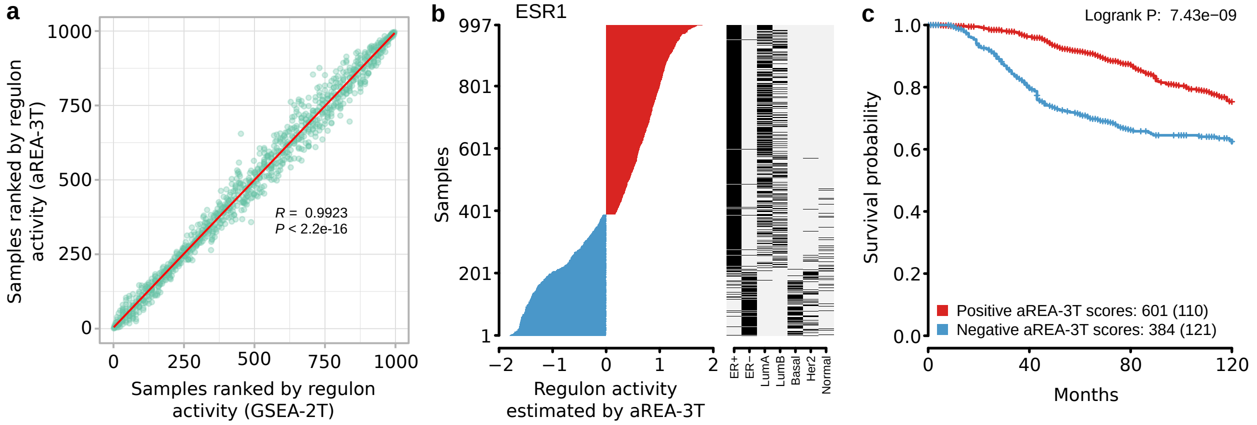
There are other tools that provide computational infrastructure to explore regulatory networks. Lefebvre *et al.* (2010) and Tarca *et al.* (2009) developed competing methods to infer sample-specific activities of curated pathways, called *PARADIGM* (PAthway Recognition Algorithm using Data Integration on Genomic Models) and *SPIA* (Signaling Pathway Impact Analysis), respectively. Both approaches predict pathway activities in a sample using gene expression and/or other genomic data (*e.g.* copy number alterations). One essential aspect of these approaches is that they have been designed to assess activity of curated pathways, usually represented by sets of genes annotated in a peer-reviewed process dedicated to provide understanding on, *e.g.* cells, organisms and ecosystems. Currently a large number of resources provide reference pathway annotation, for example, *KEGG* (Kanehisa *et al.*, 2016), *Reactome* (Fabregat *et al.*, 2018), *PID* (Schaefer *et al.*, 2009), *Gene Ontology* (The Gene Ontology Consortium, 2017) and *MSigDB* (Liberzon *et al.*, 2015), the latter representing gene set collections that encompass various other curated pathway resources. However, neither of these approaches is designed to reconstruct TF-centric regulons for a tissue of interest, and neither calculates regulon activity on an individual sample basis. To our knowledge, only *RTN* (Castro *et al.*, 2016; Fletcher *et al.*, 2013) and *VIPER* (Alvarez *et al.*, 2016) provide computational infrastructure for that purpose, both tools using the same principles as the *MARINa* algorithm (Lefebvre *et al.*, 2010), which is inspired by the two-tailed GSEA (Lamb *et al.*, 2006). Alvarez *et al.* (2016) compared 12 regulon activity metrics and concluded that the three-tailed analytic Rank-based Enrichment Analysis (aREA-3T) algorithm provides better accuracy and specificity in detecting changes in protein activity after genetic perturbations, closely followed by GSEA-2T. Both GSEA-2T and aREA-3T algorithms are available in *RTNsurvival* for sorting samples in a cohort. **Supplementary Figures 3a,b** show GSEA-2T results for the ESR1 regulon. To calculate similar results using aREA-3T:

```
#-- Compute regulon activity for individual samples using aREA-3T algorithm
tns1st_area <- tnsAREA3(tns1st)
```

```
#-- Sort sample by regulon activity estimated by aREA-3T and GSEA-2T algorithms
regact_area <- tnsGet(tns1st_area, "regulonActivity")$dif
r_gsea <- apply(regact_gsea, 2, rank)
r_area <- apply(regact_area, 2, rank)
plot(r_gsea[,"ESR1"], r_area[,"ESR1"])
```

```
#-- Compute regulon activity for individual samples using aREA-3T algorithm
tns1st_area <- tnsKM(tns1st_area)
tnsPlotKM(tns1st_area, regs = "ESR1", attribs = attribs, panelWidths=c(3,1,4), width = 6)
```

Supplementary Figure 4a shows that aREA-3T and GSEA-2T algorithms are highly concordant in sorting samples by ESR1 regulon activity. **Supplementary Figures 4b,c** show a KM analysis run by RTNsurvival using aREA-3T (compare to Supplementary Figures 3a,b). As the regulon activity scores from the current aREA-3T implementation follow a more continuous distribution than those from GSEA-2T, aREA-3T provides clearer boundaries to stratify the cohort into *pos* vs. *neg* groups, but less-clear boundaries to assign the *undetermined* group; therefore the cohort is simply divided into two groups with positive and negative aREA scores.



Supplementary Figure 4: Concordance between aREA-3T and GSEA-2T algorithms in sorting samples in a cohort. (**a**) The scatter plot shows METABRIC cohort 1 samples (n=997) sorted by ESR1 regulon activity estimated by aREA-3T (y-axis) and GSEA-2T algorithms (x-axis). (**b**) Left: stratification by ESR1 regulon activity (estimated by aREA-3T) of all 997 samples in METABRIC cohort 1. Right: ER status and PAM50 subtypes. (**c**) Kaplan-Meier survival curves for the groups highlighted in (b). Numbers indicate patients in each group and, in curved parentheses, deceased patients.

2. TCGA hepatocellular carcinoma cohort (TCGA-LIHC)

2.1 Context

In **section 1**, we used a precalculated transcriptional network for the METABRIC breast cancer cohort, which we made available as the *Fletcher2013b* data package. In **section 2**, we work with a TCGA cohort. We walk through how to use *RTN* and *RTNsurvival* with harmonized GRCh38/hg38 RNA-seq data, which we download from the Genomic Data Commons (GDC, https://gdc.cancer.gov) with the *TCGAbiolinks* package (Colaprico *et al.*, 2016). We combine the gene expression data with the cohort's molecular and clinical data, which we download from the The Cancer Genome Atlas Research Network (2017) supplements. We use outcomes data that we download from the Cell web site for the Pan-Cancer Atlas clinical data publication (Liu *et al.*, 2018). We show how to calculate the network from this data with *RTN*, then how to perform outcome analysis with *RTNsurvival*. Our goals are similar to those in **section 1**.

2.2 Download pre-processed data

To run *RTNsurvival* for a new cohort, we need a gene expression matrix for the cohort, a list of transcriptional factors, and patient metadata from the cohort. The patient metadata may consist solely of some outcome — *e.g.* overall survival (OS), progression-free interval (PFI), disease-free interval (DFI). While the patient information must be include at least two variables, `time` and `event`, it may also contain more information that can be used as attributes and covariates in *RTNsurvival* functions.

First, we'll download the pre-processed `SummarizedExperiment` object. All the preprocessing steps, from the initial GDC download to the final object, are available on the `csgroen/RTN_example_TCGA_LIHC` repository on Github. The downloaded object consists of three main components: a gene expression matrix, a patient metadata data frame and a gene metadata data frame. We will also get a separate object that contains a list of transcription factors with the necessary annotation.

First, we'll download the pre-processed `SummarizedExperiment` object. All the preprocessing steps, from the initial GDC download to the final object, are available on the `csgroen/RTN_example_TCGA_LIHC` repository on Github. The downloaded object consists of three main components: a gene expression matrix, a patient metadata data frame and a gene metadata data frame. We will also get a separate object that contains a list of transcription factors with the necessary annotation.

```r
#-- Repository link and file names
repo_link <- "https://github.com/csgroen/RTN_example_TCGA_LIHC/raw/master/"
fname_exp <- "tcgaLIHCdata_preprocessed.RData"
fname_tfs <- "tfEnsembls.RData"

#-- Download TCGA LIHC data
download.file(paste0(repo_link, fname_exp), fname_exp)
load(fname_exp)

#-- Download transcription factor list and pre-process
download.file(paste0(repo_link, fname_tfs), fname_tfs)
load(fname_tfs)
```

```r
#-- Call libraries
library(RTNsurvival)
library(SummarizedExperiment)
```

2.3 Inference of the regulatory network with RTN

The *RTN* pipeline starts with the construction of a `TNI-class` object, using the `tni.constructor` method. This method takes in a matrix of gene expression and metadata on the samples and genes, as well as a vector of the regulators to be evaluated. Here, the expression matrix and metadata are available as a `SummarizedExpression` object.

```
#-- TNI constructor
lihcTNI <- tni.constructor(tcgaLIHCdata, regulatoryElements = tfEnsembls)
```

This method also performs pre-processing to check the consistency of all the given arguments and to maximize algorithm performance. It returns a TNI (Transcriptional Network - Inference) object. The next steps run the *RTN* pipeline to generate the regulons (please refer to Fletcher *et al.* (2013), Castro *et al.* (2016) and Robertson *et al.* (2017) for additional details). To run in multithreaded mode, we suggest looking at the `tni.permutation` and `tni.boostrap` documentation.

```
#-- RTN pipeline
#-- Note: this may take some time; for multithreaded mode, please see
#-- 'tni.permutation' or 'tni.bootstrap' documentation
lihcTNI <- tni.permutation(lihcTNI, pValueCutoff = 10^-5, estimator = "spearman")
lihcTNI <- tni.bootstrap(lihcTNI, nBootstraps = 200)
lihcTNI <- tni.dpi.filter(lihcTNI)
```

The `tni.regulon.summary` method lets us get information about the regulons reconstructed by our network. For most calculations, we'll use the DPI-filtered network, which is enriched with direct regulation relationships. From the summary below, we see that the median regulon size is 30 targets and the mean size is about 49, and, while most regulons in the network will be small, some regulons have over 400 targets.

```
tni.regulon.summary(lihcTNI)
```

```
## This regulatory network comprised of 807 regulons.

## -- DPI-filtered network:

## regulatoryElements            Targets              Edges
##                807              17709              39425
##    Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
##     0.0    12.0    30.0    48.9    64.0   434.0

## -- Reference network:

## regulatoryElements            Targets              Edges
##                807              17709            1646659
##    Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
##       0     137    1376    2040    3622    7807
## ---
```

2.4 Univariate and multivariate survival analyses with *RTNsurvival*

For the survival analysis, we'll define Age and Tumour Stage as covariates for the Cox regression and evaluate 5-year (60 months) overall survival (OS).

```
#-- RTNsurvival pipeline
lihcTNS <- tni2tnsPreprocess(lihcTNI,
                             time = "OS.time.months", event = "OS",
                             endpoint = 60, keycovar = c("Age", "Tumor_Stage"))
lihcTNS <- tnsGSEA2(lihcTNS)
lihcTNS <- tnsKM(lihcTNS)
lihcTNS <- tnsCox(lihcTNS)
```

We can explore the Kaplan-Meier and Cox model results compactly in tables.

```
#-- Explore results
head(tnsGet(lihcTNS, "kmTable"), 10)
```

Table 2: Top 10 regulons in survival curve differences (G-rho test).

Regulons	ChiSquare	Pvalue	Adjusted.Pvalue
FUBP1	35.91304	0.0e+00	0.0000012
TAL1	34.36671	0.0e+00	0.0000013
YBX1	30.82942	0.0e+00	0.0000053
E2F6	29.10570	1.0e-07	0.0000096
HMGA1	32.48896	1.0e-07	0.0000099
ENO1	31.71557	1.0e-07	0.0000107
GMEB1	27.80588	1.0e-07	0.0000107
ETV5	25.72268	4.0e-07	0.0000276
TBX19	23.25694	1.4e-06	0.0000883
TSC22D4	22.56147	2.0e-06	0.0001142

```
head(tnsGet(lihcTNS, "coxTable"), 10)
```

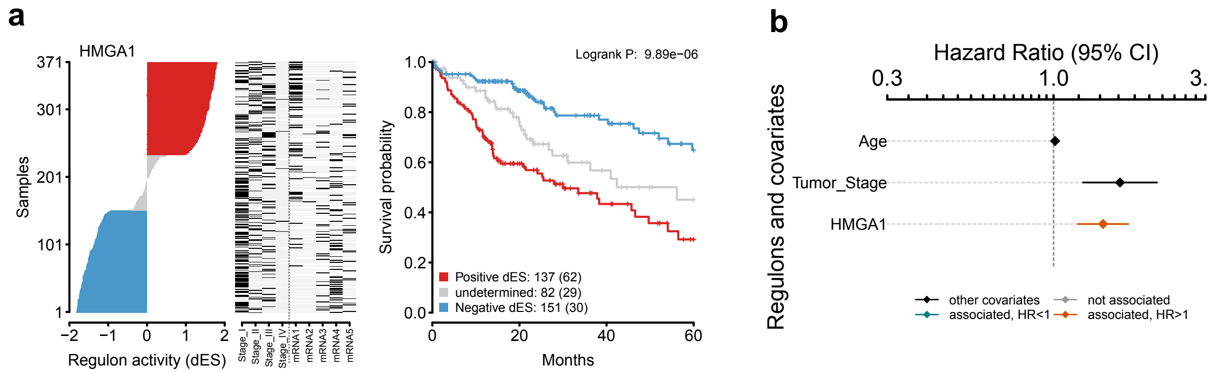
Table 3: Top 10 regulons in Cox Proportional Hazards model.

Regulons	HR	Lower95	Upper95	Pvalue	Adjusted.Pvalue
FUBP1	2.1408242	1.4948719	3.0659003	4.00e-07	0.0002328
YBX1	2.0069439	1.4249945	2.8265538	1.20e-06	0.0003319
HMGA1	1.4307089	1.1916198	1.7177693	2.90e-06	0.0004085
E2F6	2.0915900	1.4349031	3.0488112	2.90e-06	0.0004085
TAL1	0.4488133	0.2940245	0.6850903	6.00e-06	0.0006811
GMEB1	1.9245884	1.3521668	2.7393368	9.40e-06	0.0007730
ZNF408	1.7870618	1.3063348	2.4446946	9.60e-06	0.0007730
Tumor_Stage	1.6178076	1.2370032	2.1158406	1.85e-05	0.0012039
KLF9	0.7333000	0.6161108	0.8727795	2.09e-05	0.0012039
E2F5	1.8708093	1.3156337	2.6602598	2.14e-05	0.0012039

The `tnsPlotKM` method can provide a more complete picture, showing the dynamic range of the activity of a regulon, and how other variables (*e.g.* Stage, mRNA subtypes) are distributed when the cohort is ordered by activity. In this example, we use Tumor Stage and mRNA-cluster membership (only available for the 196 *core* tumour samples, see TCGA, 2017) to get an idea of how samples with low and high HMGA1 activity differ.

```
#-- Kaplan-Meier panel
tnsPlotKM(lihcTNS, "HMGA1",
          attribs = list(c("Stage_I", "Stage_II", "Stage_III", "Stage_IV"),
                         c("mRNA1", "mRNA2", "mRNA3", "mRNA4", "mRNA5")),
          panelWidths = c(2,1,3))
#-- Cox multivariate plot
tnsPlotCox(lihcTNS, "HMGA1", ylab = "Regulons and covariates")
```

The left-most panel of **Supplementary Figure 5a** shows the distribution of HMGA1 regulon activity in the cohort tumours, with low activity at the bottom and high activity at the top. The same order is used for



Supplementary Figure 5: Regulon-based survival analysis for HMGA1 in TCGA-LIHC. (**a**) Three-panel Kaplan-Meier plot for HMGA1. Left: ranking of regulon activity in the samples; Center: Stage and mRNA-cluster covariates along the samples; Right: Kaplan-Meier curve for regulon activity strata. (**b**) Cox multivariate analysis with covariates Stage, and Age and HGMA1 regulon activity.

the covariate tracks in the center panel, showing tumour stage and mRNA cluster. Given the distribution of the tumours, Stage is an interesting covariate for the Cox model. From **Supplementary Figure 5b**, we see that even when evaluated with Age and Stage, HMGA1 is still informative of survival and linked to increased hazard. In this model, each unit increase in HMGA1's regulon activity corresponds to a 43% higher hazard.

High mobility group A proteins are chromatin remodelers (Sgarra *et al.*, 2018). HMGA1 overexpression induces oncogenesis and metastasis in cultured cell lines of many phenotypes (Sumter *et al.*, 2016). Indeed, its overexpression is also linked to poorer prognostic is several cancer types, including hepatocarcinoma (Chang *et al.*, 2005) (Andreozzi *et al.*, 2016).

For the regulon activity metric, we don't consider the expression of the gene itself, only of its inferred targets; hence, it's a measure of how active a regulator is in a given tumour, not of the regulator's expression in that tumour. Here, we show that in addition to HMGA1's expression being a prognostic marker (see above publications), its regulon activity is also associated with poorer outcomes.

3. Conclusions and perspectives

RTNsurvival extends the functionality of the *RTN* package by finding regulons that are associated with outcomes like survival or progression. The regulon survival analysis uses information about the state of the regulon (*i.e.* the targets of a regulator) to find these associations.

In these examples, we have used transcription factors as examples of regulators. Transcription factors are particularly well-suited for transcriptional networks, but any regulators whose effect can be reliably measured at the transcriptional level can be used by *RTN* and *RTNsurvival*.

While the multivariate analysis provided by the package considers covariates of the user's choice, its default analysis it considers only one regulon at the time with these covariates. (*e.g.* **Supplementary Figure 5b**) For a multivariate survival analysis that considers covariates and more than one regulon at a time, the regulon activity and all relevant covariates can be recovered from the `TNS-class` object, as follows.

```r
#-- Get data and bind
full_survData <- tnsGet(lihcTNS, "survivalData")
regulon_activity <- tnsGet(lihcTNS, "regulonActivity")$dif
lihc_data <- cbind(full_survData, regulon_activity)

#-- Example Cox with multiple regulons (FUBP1 and HMGA1)
library(survival)
coxph(Surv(time, event) ~ Tumor_Stage + HMGA1 + FUBP1, data = lihc_data)
```

```
## Call:
## coxph(formula = Surv(time, event) ~ Tumor_Stage + HMGA1 + FUBP1,
##     data = lihc_data)
##
##                coef exp(coef) se(coef)      z        p
## Tumor_Stage  0.5053    1.6574   0.1038  4.869 1.12e-06
## HMGA1        0.1887    1.2077   0.0906  2.083   0.0372
## FUBP1       -0.3651    0.6941   0.1987 -1.837   0.0662
##
## Likelihood ratio test=27.87  on 3 df, p=3.874e-06
## n= 346, number of events= 116
##    (25 observations deleted due to missingness)
```

This approach can also be used for more complex survival models, such as LASSO, Adaptive LASSO, Elastic net and others. A LASSO approach was used by Robertson *et al.* (2017) to identify regulons and other covariates linked to outcome in bladder cancer. R packages `hdnom` (Xiao *et al.*, 2016) and `caret` (Kuhn, 2008) provide frameworks for these models.

The current implementation of *RTNsurvival* accepts only regulons identified by *RTN*; for a new cohort we recommend computing regulons with *RTN* (see **section 2**).

Given an *RTN* transcriptional network for a cohort, *RTNsurvival* allows a user to 1) estimate the regulon activity of individual samples, 2) generate regulon activity profiles across a cohort, 3) do univariate and multivariate analyses to associate regulon activity with time-to-event (*i.e.* outcomes) data. Current applications include: 1) assessing covariates across a cohort that has been sorted by regulon activity (Robertson *et al.*, 2017), 2) segregating a cohort for outcomes analysis (Robertson *et al.*, 2017) (Castro *et al.*, 2016), 3) assessing differences between subtypes (Kamoun *et al.*, 2018), and 4) assessing homogeneity/heterogeneity within a subtype (Robertson *et al.*, 2017).

The methods implemented in *RTNsurvival* can also be used with large-scale epigenomic data. For example, recently we showed that regulon activity profiles were consistent with ATAC-seq chromatin accessibility of distal enhancers in breast cancer (Corces *et al.*, 2018). This result provides additional support for regulon activities being a functional readout.

Session information

```
## R version 3.5.2 (2018-12-20)
## Platform: x86_64-pc-linux-gnu (64-bit)
## Running under: Ubuntu 18.04.1 LTS
##
## Matrix products: default
## BLAS: /usr/lib/x86_64-linux-gnu/openblas/libblas.so.3
## LAPACK: /usr/lib/x86_64-linux-gnu/libopenblasp-r0.2.20.so
##
## attached base packages:
## [1] stats     graphics  grDevices utils     datasets  methods   base
##
## other attached packages:
##  [1] survival_2.43-1      pheatmap_1.0.10      RTNsurvival_1.6.0
##  [4] RTNduals_1.6.0       Fletcher2013b_1.18.0 igraph_1.2.2
##  [7] RedeR_1.30.0         RTN_2.7.2            Fletcher2013a_1.18.0
## [10] limma_3.38.3
##
## loaded via a namespace (and not attached):
##  [1] Rcpp_1.0.0                  lattice_0.20-38
##  [3] viper_1.16.0                class_7.3-15
##  [5] snow_0.4-3                  gtools_3.8.1
##  [7] digest_0.6.18               GenomeInfoDb_1.18.1
##  [9] futile.options_1.0.1        stats4_3.5.2
## [11] evaluate_0.12               e1071_1.7-0
## [13] highr_0.7                   gplots_3.0.1
## [15] zlibbioc_1.28.0             VennDiagram_1.6.20
## [17] data.table_1.11.8           gdata_2.18.0
## [19] S4Vectors_0.20.1            Matrix_1.2-15
## [21] rmarkdown_1.11              splines_3.5.2
## [23] BiocParallel_1.16.2         stringr_1.3.1
## [25] mixtools_1.1.0              RCurl_1.95-4.11
## [27] munsell_0.5.0               DelayedArray_0.8.0
## [29] compiler_3.5.2              xfun_0.4
## [31] pkgconfig_2.0.2             BiocGenerics_0.28.0
## [33] segmented_0.5-3.0           htmltools_0.3.6
## [35] SummarizedExperiment_1.12.0 GenomeInfoDbData_1.2.0
## [37] IRanges_2.16.0              matrixStats_0.54.0
## [39] MASS_7.3-51.1               bitops_1.0-6
## [41] grid_3.5.2                  gtable_0.2.0
## [43] magrittr_1.5                formatR_1.5
## [45] scales_1.0.0                minet_3.40.0
## [47] KernSmooth_2.23-15          stringi_1.2.4
## [49] XVector_0.22.0              futile.logger_1.4.3
## [51] lambda.r_1.2.3              RColorBrewer_1.1-2
## [53] tools_3.5.2                 Biobase_2.42.0
## [55] parallel_3.5.2              yaml_2.2.0
## [57] colorspace_1.3-2            GenomicRanges_1.34.0
## [59] caTools_1.17.1.1            knitr_1.21
```

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