



UNIVERSITY OF SÃO PAULO  
RIBEIRÃO PRETO MEDICAL SCHOOL (FMRP)

*DOCTORAL THESIS*

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Development and enhancement of bioinformatic tools to integrate and understand aberrant epigenomic and genomic changes associated with cancer

*Methods, development and analysis*

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By

TIAGO CHEDRAOUI SILVA

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Development and enhancement of bioinformatic tools to integrate  
and understand aberrant epigenomic and genomic changes  
associated with cancer

**Tiago Chedraoui Silva**

*Advisor: Prof. Dr. Houtan Noushmehr*

Doctoral dissertation submitted to the *Ribeirão Preto Medical School* (FMRP/USP), in partial fulfillment of the requirements for the degree of the Doctorate Specialization in Normal and Neoplastic Cell Differentiation. *FINAL VERSION.*

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*To my family who always loved me and gave me all the support to achieve my dreams.*



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*“Our greatest weakness lies in giving up.  
The most certain way to succeed is always  
to try just one more time.”*

*(Thomas A. Edison)*



# Resumo

O câncer configura uma das maiores causas de mortalidade no mundo, caracterizando-se como uma doença complexa orquestrada por alterações genômicas e epigenômicas capazes de alterar a expressão gênica e a identidade celular. Nova evidência obtida por meio de um estudo genômico em larga escala e cujos dados encontram-se disponíveis no banco público do TCGA sugere que um em cada dez pacientes portadores de câncer pode ser classificado com maior eficácia tendo como base a taxonomia molecular quando comparada à histologia. Dessa maneira, nós hipotetizamos que o estabelecimento de mapas genômicos exibindo a localização de sítios de ligação de fatores de transcrição combinada à identificação de regiões diferencialmente metiladas e perfis alterados de expressão gênica possa nos auxiliar a caracterizar e explorar, ao nível molecular, fenótipos associados ao câncer.

Avanços tecnológicos e bancos de dados públicos a exemplo do The Cancer Genome Atlas (TCGA), The Encyclopedia of DNA Elements (ENCODE) e o NIH Roadmap Epigenomics Mapping Consortium (Roadmap) têm proporcionado um recurso inestimável para interrogar o (epi)genoma de linhagens de células tumorais em cultura, bem como de tecidos normais e tumorais em alta resolução. Todavia, a informação biológica encontra-se armazenada em diferentes formatos e não há ferramentas computacionais para integrar esses dados, evidenciando um cenário atual que requer, com urgência, o desenvolvimento de ferramentas de bioinformática e softwares capazes de direcionar a solução deste obstáculo. Nesse contexto, o objetivo principal deste estudo consiste em implementar o desenvolvimento do pacote biOMICs, o qual será codificado usando-se a licença de código aberto GNU GPL e a linguagem de programação R e que, ao final do estudo, será submetido à comunidade científica do projeto Bioconductor. Além disso, ajudaremos nossos colaboradores com o aperfeiçoamento do ELMER, um pacote R/Bioconductor que identifica elementos reguladores usando dados de expressão gênica, de metilação do DNA e análise de motivo.

Nossa expectativa é que o pacote biOMICs possa automatizar com acurácia a pesquisa, o download e a análise dos dados (epi)genômicos que se encontram atualmente disponíveis nas bases de dados públicas dos consórcios internacionais TCGA, ENCODE e Roadmap, além de integrá-los facilmente aos dados genômicos e epigenômicos gerados por pesquisadores por meio de experimentos em larga escala. Além disso, realizaremos também o processamento e a análise manual dos dados que serão automatizados pela ferramenta biOMICs, visando validar sua capacidade em descobrir assinaturas epigenômicas que possam redefinir subtipos de câncer. Por fim, usaremos biOMICs e ELMER para investigar as diferenças moleculares entre dois subgrupos de gliomas recentemente descobertos por nosso laboratório.

**Palavras-chave:** Câncer, metilação do DNA, redes reguladoras de genes, interações de cromatina, sítios de ligação do fator de transcrição, epi-genética, ferramentas computacionais.



# Abstract

SILVA, T. C.. **Development and enhancement of bioinformatic tools to integrate and understand aberrant epigenomic and genomic changes associated with cancer.** 2017. 99 f. Doctoral dissertation (Doctorate Candidate – Specialization in Normal and Neoplastic Cell Differentiation) – Ribeirão Preto Medical School (FMRP/USP), Ribeirão Preto – SP.

Cancer, which is one of the major causes of mortality worldwide, is a complex disease orchestrated by aberrant genomic and epigenomic changes that can modify gene regulatory circuits and cellular identity. Emerging evidence obtained through high-throughput genomic data deposited within the public TCGA international consortium suggests that one in ten cancer patients would be more accurately classified by molecular taxonomy versus histology. Therefore, we have hypothesized that the establishment of genome-wide maps of the de novo DNA binding motifs localization coupled with differentially methylated regions and gene expression changes might help to characterize and exploit cancer phenotypes at the molecular level.

Technological advances and public databases like The Cancer Genome Atlas (TCGA), The Encyclopedia of DNA Elements (ENCODE), and The NIH Roadmap Epigenomics Mapping Consortium (roadmap) have provided unprecedented opportunities to interrogate the epigenome of cultured cancer cell lines as well as normal and tumor tissues with high resolution. Markedly however, biological information is stored in different formats and there is no current tool to integrate the data, highlighting an urgent need to develop bioinformatic tools and/or computational softwares to overcome this challenge. In this context, the main purpose of this study is the development of biOMICs, a package coded in the GNU GPL (General Public License) R programming language that will be submitted to the larger open-source Bioconductor community project. Also, we will help our collaborators improve of the R/Bioconductor ELMER package that identifies regulatory enhancers using gene expression, DNA methylation data and motif analysis.

Our expectation is that biOMICs can effectively automate search, retrieve, and analyze the vast (epi)genomic data currently available from TCGA, ENCODE, and Roadmap, and integrate genomics and epigenomics features with researchers own high-throughput data. Furthermore, we will also navigate through these data manually in order to validate the capacity of biOMICs in discovering epigenomic signatures able to redefine subtypes of cancer. Finally, we will use biOMICs and ELMER to investigate the molecular differences between two subgroups of gliomas, one of the most aggressive primary brain cancer, recently discovered by our laboratory.

**Key-words:** Cancer, DNA methylation, gene regulatory networks, enhancers, chromatin interactions, transcription factor binding sites, epi-genetics, computational tools.



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# LIST OF ABBREVIATIONS AND ACRONYMS

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- ANOVA ... Analysis of variance  
AUC ..... Area Under the Curve  
AWGs ..... Analysis working groups  
CBS ..... circular binary segmentations  
CCG ..... Center for Cancer Genomics  
ChIP-seq . ChIP-sequencing  
CNP ..... Copy-number polymorphism  
CNV ..... copy number variation  
COAD .... Colon adenocarcinoma  
DEA ..... differential expression analysis  
DEGs ..... differentially expressed genes  
DMCs ..... differentially methylated CpGs  
DMR ..... Differentially methylated regions  
DNA ..... deoxyribonucleic acid  
ELMER .. Enhancer Linking by Methylation/Expression Relationship  
ENCODE . The Encyclopedia of DNA Elements  
EPIC ..... MethylationEPIC BeadChip  
FC ..... fold change  
FDR ..... False discovery rate  
FDR ..... false discovery rate  
FIMO .... Find Individual Motif Occurrences  
FOXA1 ... Forkhead box protein A1  
FWER .... Family-wise error rate  
GATA3 ... GATA-binding protein 3  
GDC ..... Genomic Data Commons  
GNU GPL3 GNU General Public License version 3  
GRanges . GenomicRanges  
GRCh36 .. Genome Reference Consortium Human Build 36  
GRCh37 .. Genome Reference Consortium Human Build 37  
GRCh38 .. Genome Reference Consortium Human Build 38  
GSEA .... gene set enrichment analysis

GUI ..... Graphical User Interface  
HM450 ... HumanMethylation450 BeadChip  
HOCOMOCO HOmo sapiens COmprehensive MOdel Collection  
HOMER .. Hypergeometric Optimization of Motif EnRichment  
HOXD13 . Homeobox D13  
HOXD3 .. Homeobox D3  
KW ..... Kruskal-Wallis test  
LGG ..... lower-grade glioma  
MAE ..... MultiAssayExperiment  
MAF ..... Mutation Annotation Format  
MAF ..... Mutation Annotation Format  
MWU .... Mann-Whitney U-test  
NGS ..... next-generation sequencing  
NHGRI ... National Human Genome Research Institute  
OR ..... Odds Ratio  
P63 ..... tumor protein p63  
PPIN ..... Protein-protein interaction networks  
PPIs ..... Protein-protein interactions  
PWM .... position weight matrix  
qCML .... quantile-adjusted conditional maximum likelihood  
ROADMAP The NIH Roadmap Epigenomics Mapping Consortium  
ROC ..... Receiver Operator Characteristics  
SE ..... SummarizedExperiment  
SNP ..... single nucleotide polymorphism  
SOX2 .... Sex determining region Y-box 2  
TARGET . Therapeutically Applicable Research to Generate Effective Treatments  
TCGA .... The Cancer Genome Atlas  
TF ..... Transcription Factor  
TFBSs ... Transcription factor binding sites  
TSSs ..... transcription start sites  
WGBS ... whole-genome bisulfite sequencing  
WRST ... Wilcoxon Rank Sum Test

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## LIST OF SYMBOLS

$H_0$  — Null hypothesis

$H_1$  — Alternative hypothesis

$\alpha$  — Significance level

$\beta$  — rate of the type II error

$S(t)$  — Kaplan–Meier estimator



# CONTENTS

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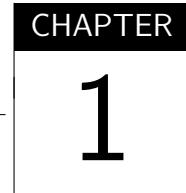
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# INTRODUCTION

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## 1.1 Objectives

The main goal of this project is to develop tools for searching, retrieving and analyzing pan-cancer genomic data from several databases, such as the NCI's Genomic Data Commons (GDC), which contains data from The Cancer Genome Atlas (TCGA) and Therapeutically Applicable Research to Generate Effective Treatments (TARGET), The Encyclopedia of DNA Elements (ENCODE), and The NIH Roadmap Epigenomics Mapping Consortium (ROADMAP). For a better transparency, all tools will be open source and for their scalability and interoperability they will be published in the Bioconductor project, an environment that provides a broad range of powerful statistical and graphical methods for the analysis of genomic data. Furthermore, we also aim to investigate the intergenic epigenomic changes associated with distinct biological and clinical subgroups of gliomas first discovered by our laboratory (CECCARELLI *et al.*, 2016b). Specifically, using the tools developed, we will integrate DNA methylation, gene expression, mutation and copy number data as well as important epigenomic marks defined by histone modifications in normal samples in order to identify candidate regulatory elements associated with glioma progression.

### 1.1.1 *Specific aims*

1. Download and process transcription factor (TF) ChIP-seq data for each cancer cell and tissue type through the ENCODE dataset;
2. Download and process DNA methylation data for both cancer and non-tumor control cases through the TCGA consortium via HM450K platform;
3. Identify statistically Differentially methylated regions (DMR) at the single CpG resolution;
4. Determine statistically enriched proximal transcription factor binding sites (TFBSs) to altered DNA-methylated regions at the level of individual DNA/protein site interaction;

5. Within known DMRs, classify and identify statistically known and novel DNA binding motifs;
6. Download and process RNA-seq data from both cancer and non-tumor control cases
7. Use standard data structure to organize the data and the metadata;
8. Correlate the DNA methylation status of Transcription factor binding sites (TFBSs) with target gene RNA-seq expression in order to determine regulatory networks that might alter the pan-cancer genome;
9. Use learning machine algorithms for classifying an independent set of gliomas based on newly identified regulatory networks as related to pan-cancer deregulation;
10. Develop tools to automate the previous steps;
11. Use those tools to investigate the intergenic epigenomic changes associated with distinct biological and clinical subgroups of gliomas g-cimp-low and g-cimp-high discovered by our laboratory and collaborators;
12. Compare the automated results with ones found manually in order to validate the package capacity in providing searching, retrieving and downstream biological analysis to discover pan-cancer epigenomic signatures able to redefine subgroups of gliomas;
13. Submit those set of tools to be freely available at the open-source Bioconductor environment (available at <http://www.bioconductor.org>).

## 1.2 Motivations

Unravelling the genomic, epigenomic, and proteomic features using high-throughput methodologies is a central question for understanding regulatory gene networks in cancer. However, a major challenge is the fact that the biological information necessary for a complete gene regulatory analysis is spread over different databases that store data in different formats. Moreover, to date there is no current computational tool that can integrate and interpret such information. Consequently, the process of analysis is performed manually by the end user, who must access all databases, select and process the data necessary to the project, and integrate that data using multiple downstream analysis tools to extract and interpret the relevant biological information.

Recently, to address this need, our laboratory published the open-source software package known as [TCGAbiolinks](#) (COLAPRICO *et al.*, 2016) that automates the retrieval, assembly, and processing of public TCGA data. Also, laboratory of Dr Benjamin Berman published an open-source software package known as [ELMER](#) (YAO *et al.*, 2015) that identifies regulatory enhancers using gene expression, DNA methylation data and motif analysis. Although both tools

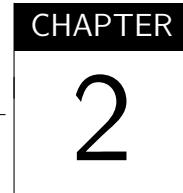
are of utmost importance, there is still the need to create a more complete tool that can work with more data from different platforms and new methods of analysis.

To overcome this challenge, we are creating a bioinformatic tool to automate the retrieval, assembly, processing and analysis of data that are publicly deposited by international consortia such as TCGA, ENCODE and Roadmap and we will help on the improvement of the ELMER package. Finally, we will use these tools to perform molecular analysis between the newly identified groups G-CIMP-low and G-CIMP-high and understand tumor genesis and the tumorigenic process.

### 1.3 Organization of the Remainder of the Document

This paper is organized in chapters as follows. In Chapter 2, we introduce the fundamental concepts required to the development of this project. With this respect, we review the basic definitions genetics and epigenetics area. After presenting the theoretical concepts required to understand the work, we present the results of the work in Chapters 3, 4, and 5. Specifically, in Chapter 3, we explore the methods and computational tools developed. In Chapter 4, we show the results of the use of the methods and tools presented in chapter 3 another real-world application. Enclosing this thesis, in Chapter 5, we draw the main conclusions of this work. Moreover, the scientific contributions derived from this project are listed, as well as possible future works. Lastly, we show the main papers that have been published during the Doctorate period.





# REVIEW OF ESSENTIAL CONCEPTS

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This chapter introduces the fundamental concepts related to the developments presented in this thesis. The major areas introduced are: Cancer, which briefly describes some of their characteristics, Section 2.1 introduces cancer, its characteristics when compared to normal cells, the molecular mechanisms responsible for its onset and those that facilitate its maintenance and proliferation. Section 2.2 focuses on presenting more details of the genetic and epigenetic changes in cancer cells. Section 2.3 describes some consortia that publicly made available clinical, genetic and epigenetic data and how this data is computationally represented and structured. Section 2.4 presents several techniques of data analysis used to extract biological knowledge of this data.

## 2.1 Cancer

Tumors are complex tissues composed of multiple distinct cell types that participate in heterotypic interactions with one another. For the onset of tumorigenesis, cancer cells create a "tumor microenvironment" recruiting normal cells which are active participants in tumorigenesis rather than passive bystanders. If compared to normal cells, cancer cells acquired capabilities which includes sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction. Some examples of these acquired capabilities are described below.

**Sustaining Proliferative Signaling** While normal tissues carefully control the production and release of growth-promoting signals the cancer cells deregulates these signals in ways that promote proliferation. Cancer cells can acquire the capability to sustain proliferative signaling in a number of alternative ways: They may produce growth factor ligands themselves, they may send signals to stimulate normal cells within the supporting tumor-associated stroma, to supply them with various growth factors (CHENG *et al.*, 2008),

or they may elevate the levels of receptor proteins displayed at the cancer cell surface, rendering such cells hyperresponsive to otherwise-limiting amounts of growth factor ligand. Cancer cell also disrupts Negative-Feedback Mechanisms that Attenuate Proliferative Signaling, for example, loss-of-function mutations or promoter methylation of the PTEN, a tumor suppressor, amplify PI3K signaling and promote tumorigenesis in a variety of experimental models of cancer (JIANG; LIU, 2009). A second example is the oncogene RAS that some mutations affecting it compromise Ras GTPase activity, a protein that normally regulate the inactivation of Ras. In addition to the oncogenic mutations in Ras, another mechanism affecting this gene, showed silence of the regulatory Ras GTPase proteins through CpG methylation (JIN *et al.*, 2007).

**Evading growth suppressors** Cancer cells must also circumvent actions of tumor suppressor genes that negatively regulate cell proliferation. One example of tumor suppressor proteins is the RB (retinoblastoma-associated), that integrates signals from diverse extracellular and intracellular sources and, in response, decides whether or not a cell should proceed through its growth-and-division cycle (BURKHART; SAGE, 2008). Cancer cells with defects in RB pathway function are thus missing the services of a critical gatekeeper of cell-cycle progression whose absence permits persistent cell proliferation. Another example, is the TP53 proteins that receives inputs from stress and abnormality sensors that function within the cell's intracellular operating systems. This protein can activate apoptosis if it received a signal of irreparable damage to the cellular subsystems, or it can interrupt the cell-cycle progression if it receives signals that the degree of damage to the genome is excessive, or if the levels of nucleotide pools, growth-promoting signals, glucose, or oxygenation are suboptimal (HANAHAN; WEINBERG, 2011). Moreover, disruption of such self attenuating signaling may contribute to the development of adaptive resistance toward drugs targeting mitogenic signaling. To evade those growth suppressors proteins tumor cells developed mechanisms of contact inhibition and its evasion

**Resisting Cell Death** The loss of TP53 tumor suppressor function, which eliminates this critical damage sensor from the apoptosis-inducing circuitry, is a tumor cell strategies to limit or circumvent apoptosis (HANAHAN; WEINBERG, 2011).

Those capabilities, which enable tumor growth and metastatic dissemination, were acquired mainly due to a genetic diversity generated by genome instability (HANAHAN; WEINBERG, 2011). A multistep tumor progression can be portrayed as a succession of clonal expansions, which can be triggered by nonmutational changes (epigenetic mechanisms such as DNA methylation and histone modifications (BERDASCO; ESTELLER, 2010)) or mutational changes affecting the regulation of gene expression.

To increase the rates of mutation, cancer cells often breakdown components of the genomic maintenance machinery that normally monitor genomic integrity and force genetically

damaged cells into either senescence or apoptosis (JACKSON; BARTEK, 2009), more specifically those involved in detecting DNA damage and activating the repair machinery, in directly repairing damaged DNA, and in inactivating or intercepting mutagenic molecules before they have damaged the DNA (NEGRINI; GORGOULIS; HALAZONETIS, 2010). Another major source of tumor-associated genomic instability is the loss of telomeric DNA in many tumors that generates karyotypic instability and associated amplification and deletion of chromosomal segments ???. Recurrence of these amplifications and deletions aberrations at particular sites in the genome indicates that they are likely to harbor genes whose alteration favors neoplastic progression (KORKOLA; GRAY, 2010).

## 2.2 Genetics and epigenetics alterations

While genetics is the study of heritable changes in gene activity or function due to the direct alteration of the DNA sequence such as point mutations, deletions, insertions, and translocation, epigenetics is the study of those not associated with any change of the DNA sequence (MOORE; LE; FAN, 2013). Even though all cells in an organism contain the same genetic information, this epigenetic mechanism controls which gene are expressed enabling the existence of a diversified gene expression profiles in a variety of cells and tissues in multicellular organisms.

### 2.2.1 DNA methylation

DNA methylation is an epigenetic mechanism involving the transfer of a methyl group onto the C5 position of the cytosine to form 5-methylcytosine, which regulates gene expression by either blocking the binding of Transcription factors or recruiting proteins involved in gene repression (MOORE; LE; FAN, 2013). The pattern of DNA methylation in the genome can change by DNA methylation and demethylation process, which are process involved in cell differentiation as unique DNA methylation pattern that regulates tissue-specific gene transcription (MOORE; LE; FAN, 2013).

The DNA methylation process is mediated by the family of DNA methyltransferases (Dnmts) proteins which catalyzes the transfer of a methyl group from S-adenyl methionine (SAM) to the fifth carbon of cytosine residue to form 5-methylcytosine (5mC). The main proteins responsible for this transfer are the Dnmt3a and Dnmt3b, which are involved in establishing a new methylation pattern to unmodified DNA, and Dnmt1, which is responsible to the methylation of a daughter strand during the DNA replication process.

The majority of DNA methylation occurs on cytosines that precede a guanine nucleotide (CpG sites), however Xie *et al.* (2012) reported a significant percentage of methylated non-CpG sites (XIE *et al.*, 2012).

The role of DNA methylation varies in different genomic regions. Within intergenic regions the DNA methylation repress the expression of potentially harmful genetic elements, while within promoter regions containing CpG islands, stretches of DNA roughly 1000 base pairs long that have a higher CpG density than the rest of the genome and often are not methylated (BIRD *et al.*, 1985), results in stable silencing of gene expression, results in stable silencing of gene expression (MOHN *et al.*, 2008). Within the gene body, region of the gene past the first exon, the methylation of the first exon leads to gene silencing (BRENET *et al.*, 2011).

## 2.2.2 *Histone modifications*

This process of transcription regulation involving DNA methylation, works in association with histone modifications and noncoding RNAs.

Normally, the DNA is wrapped around the histone proteins forming small, packaged sections called nucleosomes. This association is capable of inhibiting gene expression since a more compact region hinders the accessibility of transcription factors.

Those histones might have chemical modifications (methylation, acetylation, ubiquitination, and phosphorylation) added to three specific amino acids on their N-terminal tail which influence how DNA strands are packaged and their transcriptional activity. Those modifications that loosen DNA association with histones generally provide a permissive environment for transcription, whereas the ones that tighten them repress gene expression (MOORE; LE; FAN, 2013).

These modifications involved in adding and/or stripping histone markers in order to impose a repressive state on a gene region, are led by histone-modifying enzymes and are in general in cooperation with the Dnmts proteins which are involved in the mechanism of DNA methylation. For example, Dnmt1 and Dnmt3 are known to bind to the histone methyltransferase SUV39H1 that restricts gene expression by methylation on H3K9 (FUKS *et al.*, 2003). Another example are Dnmt1 and Dnmt3b that can both bind to histone deacetylases that remove acetylation from histones to make DNA pack more tightly and restrict access for transcription (FUKS *et al.*, 2000; GEIMAN *et al.*, 2004). Also, H3K36 trimethylation, a repressive histone mark, stimulates the methyltransferase activity, the H3K4 trimethylation (H3K4me3) prevents it (OOI *et al.*, 2007; ZHANG *et al.*, 2010).

The miRNAs have emerged as another important epigenetic mechanism that influences gene expression. It can repress gene expression by inhibiting translation or inducing RNA degradation (BEREZIKOV, 2011) and it not only can regulate histone modifications and Dnmt expression thus regulating DNA methylation (BENETTI *et al.*, 2008; SINKKONEN *et al.*, 2008), but also the DNA methylation can regulate the expression of miRNAs (HAN *et al.*, 2007; LUJAMBIO *et al.*, 2008).

Overall, miRNAs, DNA methylation and histone modifications work closely together to

regulate gene expression (MOORE; LE; FAN, 2013).

### **2.2.3 *Genetics alterations***

Although the haploid human genome consists of 3 billion nucleotides, changes in even a single base pair might result in dramatic physiological malfunctions. A mutation, defined as any alteration in the DNA sequence, can be a germ-line mutation, which occurs in a germ-line cell (one that will give rise to gametes) and can be passed to a descendant of the organism, and somatic mutations, which occurs in a somatic cell (one that develop into the body tissues) and are never transmit to their descendants (CLANCY, 2008).

A single base pair alteration that is present in at least 1% of the population is called single nucleotide polymorphism (SNP) and is generally used to refer to a normal variation (does not directly cause disease) (CLANCY, 2008).

An example of a point mutations that is able to alter proteins are the insertion or the deletion of a single base, called frameshift mutations. This change of nucleotides alters the codons (group of three nucleotides) read by the ribosomes, which affects the protein resulted during the translation process. Table 1 show a classification of translational effect of variant allele (INSTITUTE, 2017; GDC, 2017).

Table 1 – Translational effect of variant allele. (INSTITUTE, 2017)

| <b>Variant_Classification</b> | <b>Group</b>   |
|-------------------------------|--|
| Frame_Shift_Del               | Deletion that moves the coding sequence out of frame                                     |
| Frame_Shift_Ins               | Insertion that moves the coding sequence out of frame                                    |
| Missense_Mutation             | The point mutation alters the protein structure by one amino acid                        |
| Nonsense_Mutation             | A premature stop codon is created by the variant   |
| Silent                        | Variant is in coding region of the chosen transcript, but protein structure is identical |
| Splice_Site                   | The variant is within two bases of a splice site.  |
| In_Frame_Del                  | Deletion that keeps the sequence in frame  |
| In_Frame_Ins                  | Insertion that keeps the sequence in frame   |
| Translation_Start_Site        | Point mutation, insertion or deletion that overlaps the start codon                      |
| Nonstop_Mutation              | variant removes stop codon.  |
| 3'UTR                         | The variant is on the 3'UTR for the chosen transcript                                    |
| 3'Flank                       | The variant is downstream of the chosen transcript (within 3kb)                          |
| 5'UTR                         | The variant is on the 5'UTR for the chosen transcript                                    |
| 5'Flank                       | The variant is upstream of the chosen transcript (within 3kb)                            |
| IGR                           | Intergenic region. Does not overlap any transcript.                                      |
| Intron                        | The variant lies between exons within the bounds of the chosen transcript.               |
| De_novo_Start_InFrame         | New start codon is created in frame relative to the coded protein.                       |
| De_novo_Start_OutOfFrame      | New start codon is created out of frame relative to the coded protein.                   |
| RNA                           | The variant lies on one of the RNA transcripts.  |
| lincRNA                       | The variant lies on one of the lincRNAs.   |

Not only changes in DNA can occur on a single nucleotide called point mutations but they can also occur at the level of the chromosome, in which large segments of chromosomes are altered (deleted, duplicated, inverted, translocated to different chromosomes, rearranged) resulting in levels of gene expression, the complete absence of genes, or the alteration of gene sequence. Feuk, Carson e Scherer (2006) define structural variants as genomic alterations that involve segments of DNA that are larger than  $1kb$  which can be microscopic ( $> 3Mb$ ) or submicroscopic ( $1kb$  to  $3Mb$ ) variants, and also define smaller ( $< 1kb$ ) variations or polymorphisms that involve the copy-number change of a segment of DNA as insertions or deletions (indels). A type of structural variant is the copy number variation (CNV) are a segment of DNA that is  $1 kb$  or larger and is present at a variable copy number in comparison with a reference genome while Copy-number polymorphism (CNP) are CNVs that occurs in more than 1% of the population. Also, it is common to refer to changes in copy number that have arisen in somatic tissue as copy number alterations/aberrations (CNAs or SCNAs), and changes in copy number in germline cells as copy number variations (CNVs). Table 2 summarizes the types of mutations.

Table 2 – Types of DNA Mutations and Their Impact (COMISC, 2017; CLANCY, 2008; FEUK; CARSON; SCHERER, 2006)

| <b>Class of Mutation</b> | <b>Type of Mutation</b>        | <b>Description</b>  |
|--------------------------|--------------------------------|---|
| Point mutation           | Substitution                   | One base is incorrectly added during replication and replaces the pair in the corresponding position on the complementary strand  |
|                          | Insertion                      | One or more extra nucleotides are inserted into replicating DNA, often resulting in a frameshift  |
|                          | Deletion                       | One or more nucleotides is "skipped" during replication or otherwise excised, often resulting in a frameshift   |
| Chromosomal mutation     | Inversion                      | A segment of DNA that is reversed in orientation with respect to the rest of the chromosome.  |
|                          | Deletion                       | A region of a chromosome is lost, resulting in the absence of all the genes in that area  |
|                          | Duplication or low-copy repeat | A segment of DNA < 1kb in size that occurs in two or more copies per haploid genome, with the different copies sharing > 90% sequence identity.   |
|                          | Translocation                  | A change in position of a chromosomal segment within a genome that involves no change to the total DNA content. Translocations can be intra- or inter- chromosomal.   |
| Copy number variation    | Gain/Amplification             | A single-copy gain or a multi-copy amplification of the entire gene   |
|                          | Loss/Deletion                  | A loss for part or all of a coding region within the gene footprint in a single allele/copy (hemizygous deletion) or deletion for part or all of a coding region within the gene footprint in both alleles/copies (homozygous deletion). (COMISC, 2017) |

## 2.3 Databases and data structure

Recent technological developments allowed the deposition of large amounts of genomic and epigenomic data, such as gene expression, DNA methylation, and genomic localization of transcription factors, into freely available public international consortia like The Cancer Genome Atlas (TCGA), The NCI's Genomic Data Commons (GDC), The Encyclopedia of DNA Elements (ENCODE), and The NIH Roadmap Epigenomics Mapping Consortium (Roadmap) (HAWKINS; HON; REN, 2010). Subsection 2.3.1 presents an overview of those consortia and subsection 2.3.2 presents some of the computational data formats and data structures used to represent the biological data.

### 2.3.1 Databases

**The Cancer Genome Atlas (TCGA):** The TCGA consortium, which was a National Institute of Health (NIH) initiative, made publicly available molecular and clinical information for more than 30 types of human cancers including exome (variant analysis), single nucleotide polymorphism (SNP), DNA methylation, transcriptome (mRNA), microRNA (miRNA) and proteome . Sample types available at TCGA are: primary solid tumors, recurrent solid tumors, blood derived normal and tumor, metastatic, and solid tissue normal (WEINSTEIN *et al.*, 2013). This project was shutdown in mid 2016 and its data was moved to the NCI Genomic Data Commons (GDC).

**The Genomic Data Commons (GDC):** The NCI's Genomic Data Commons (GDC) provides the cancer research community with a unified data repository that enables data sharing across cancer genomic studies in support of precision medicine. It supports several cancer genome programs at the NCI Center for Cancer Genomics (CCG), including The Cancer Genome Atlas (TCGA) and Therapeutically Applicable Research to Generate Effective Treatments (TARGET) (NCI, 2017d). Those data is harmonized against GRCh38 (hg38) using GDC Bioinformatics Pipelines which provides methods to the standardization of biospecimen and clinical data, the re-alignment of DNA and RNA sequence data against a common reference genome build GRCh38, and the generation of derived data. Also, this repository has a Legacy Archive to provide access to an unmodified copy of data that was previously stored in CGHub (WILKS *et al.*, 2014) and in the TCGA Data Portal hosted by the TCGA Data Coordinating Center (DCC), in which uses as references GRCh37 (hg19) and GRCh36 (hg18).

**The Encyclopedia of DNA Elements (ENCODE):** Found in 2003 by the National Human Genome Research Institute (NHGRI), the project aims to build a comprehensive list of functional elements that have an active role in the genome, including regulatory elements that govern gene expression. Biosamples includes immortalized cell lines, tissues, primary cells and stem cells (CONSORTIUM *et al.*, 2011).

**The NIH Roadmap Epigenomics Mapping Consortium:** This was launched with the goal of producing a public resource of human epigenomic data in order to analyze biology and disease-oriented research. Roadmap maps DNA methylation, histone modifications, chromatin accessibility, and small RNA transcripts in stem cells and primary ex vivo tissues (FINGERMAN *et al.*, 2011; BERNSTEIN *et al.*, 2010).

Briefly, these consortia provide large scale epigenomic data onto a variety of microarrays and next-generation sequencing (NGS) platforms. Each consortium encompasses specific types of biological information on specific type of tissue or cell and when analyzed together, it provides an invaluable opportunity for research laboratories to better understand the developmental progression of normal cells to cancer state at the molecular level and importantly, correlate these phenotypes with tissue of origins.

### 2.3.2 Data and data structure

If previously the main bottleneck to scientific progress in cellular biology was data collection, now this bottleneck shifted to analysis of data (MCPHERSON, 2009). The large-scale genomic data mining, which is the process of using many diverse datasets to address a specific biological question, involves three main tasks: establishing methodology for efficiently querying large data collections; assembling data from appropriate repositories; and integrating information from a variety of experimental data types (HUTTENHOWER; HOFMANN, 2010). In data science, raw data refers to data that have not been changed since acquisition which after editing, cleaning or modifying the it results in processed data. In genomics the raw data, sequencing read data, from next generation sequencing machines is stored in a FASTQ, a common file format data that combines both the sequence and an associated per base quality score (COCK *et al.*, 2009). Those stored reads are then aligned to a reference genome sequence with software like Bowtie 2 (LANGMEAD; SALZBERG, 2012), generating SAM files, the accepted standard for storing short read alignment data, which are subsequently compressed to binary format (Binary Alignment/Map (BAM)) via SAMtools (LI *et al.*, 2009). It is common to consider this set of SAM, BAM and FASTQ files as raw data. Some databases classifies this raw data as level 1, protected level or raw level. It is important to highlight that due to privacy concerns, the access to this raw data is controlled because it could be used in identity tracing attacks (ERLICH; NARAYANAN, 2014; AYDAY *et al.*, 2014) as generally includes individually identifiable data such as low level genomic sequencing data, germline variants, SNP6 genotype data, and certain clinical data elements (NCI, 2017e). This raw data are processed through bioinformatics pipelines generating processed data files or matrices (usually in the form of tab-delimited text files). Some databases classifies this raw data as level 2 or more or open. For example, the previously stored data in CGHub, TCGA Data Portal and Broad Institute's GDAC Firehose, were provided as different levels or tiers that were defined in terms of a specific combination of both processing level (raw, normalized, integrated) and access level (controlled or open access).

Level 1 indicated raw and controlled data, level 2 indicated processed and controlled data, level 3 indicated Segmented or Interpreted Data and open access and level 4 indicated region of interest and open access data.

In the next subsections we will focus on the processed data and their data structures used in this project.

### 2.3.2.1 Data

**Array-based DNA methylation data:** DNA methylation data files contain information on raw and normalized signal intensities, detection confidence and calculated beta values for methylated and unmethylated probes. The processed data from DNA methylation array-based platforms such as GoldenGate, Infinium Human Methylation27 and the Infinium HD 450K methylation array, are presented in the form of beta-values that uses a scale ranging from 0.0 (probes completely unmethylated) up to 1.0 (probes completely methylated). In each file, one per sample, one row represents a 1 bp region (probe) and its beta-value. Also, probes overlapping with SNPs are normally masked.

**Somatic Variant data:** The DNA-Seq Somatic Variant Analysis algorithms, such as MuSE (FAN *et al.*, 2016), Mutect2 (CIBULSKIS *et al.*, 2013), SomaticSniper (LARSON *et al.*, 2011) and Varscan2 (KOBOLDT *et al.*, 2012), identify and characterizes somatic mutations by comparing reference alignments from tumor and normal samples from the same case. The results of the variant calls are stored in a Mutation Annotation Format (MAF), which are filtered to remove any potentially erroneous or germline variant calls. Each row of these represents a mutation in the genome of a sample (NCI, 2017a).

**RNA-Seq Gene Expression data :** The processed data contains read counts measured on a gene level, which might be normalized using some methods such as the Fragments Per Kilobase of transcript per Million mapped reads (FPKM) (NCI, 2017b) and FPKM Upper Quartile (FPKM-UQ) (NCI, 2017c).

**miRNA-Seq data:** The processed data contains expression levels measured and normalized post-alignment. Normalization is performed using the Reads per Millions (RPM) method. Expression levels for known miRNAs and observed miRNA isoforms are generated for each sample.

**Copy Number Variation data:** The processed data is a file with detected duplications or deletions of contiguous chromosomal regions by measuring array intensity levels. This file that might contain or not germline CN variations (files with the prefix "nocnv" had the removed). This processed data is used as input for circular binary segmentations (CBS) which divides each chromosome into contiguous segments of equal copy-number and quantifies each.

## ChIP-Seq data (Histone modification):

### 2.3.2.2 Data structures

Although there exists a wealth of possibilities (KANNAN *et al.*, 2015) in accessing cancer associated data, Bioconductor (<https://www.bioconductor.org/>) represent the most comprehensive set of open source, updated and integrated professional tools for the statistical analysis of large scale genomic data. It uses the R statistical programming language and has been developing several data structures to handle genetics, epigenetics and clinical data. Some of the mainly data strutures from the R/Bioconductor used in this project are described below.

**DataFrame:** A data frame is used for storing data tables. It is a list of vectors of equal length.

**GenomicRanges (GRanges):** This data strucute was created to representation and manipulate genomic intervals and variables defined along a genome GRanges is a vector of genomic locations, which are composed by the sequence names (chr1, chr2), the sequence range (start and end) and the strand information, and associated annotations (metadata) (LAWRENCE *et al.*, 2013). An example of GRanges object is shown in Figure 1.

```
GRanges object with 10 ranges and 2 metadata columns:
  seqnames      ranges strand |      score          GC
              <Rle>  <IRanges>  <Rle> | <integer>      <numeric>
  a      chr1 [101, 111]     - |       1             1
  b      chr2 [102, 112]     + |       2 0.8888888888888889
  c      chr2 [103, 113]     + |       3 0.7777777777777778
  .      ...   ...   ...   ... | ...
  h      chr3 [108, 118]     + |       8 0.2222222222222222
  i      chr3 [109, 119]     - |       9 0.1111111111111111
  j      chr3 [110, 120]     - |      10             0
  -----
  seqinfo: 3 sequences from an unspecified genome; no seqlengths
```

Figure 1 – Example of Granges object.

**SummarizedExperiment (SE):** This data strucute, exemplified in Figure 2, is a matrix-like container where rows represent ranges of interest (as a GRanges object) and columns represent samples (with sample data summarized as a DataFrame). It can contains one or more assays, each represented by a matrix-like object of numeric or other mode.

**MultiAssayExperiment (MAE):** This data strucute is an integrative environment where multiple assays are managed and preprocessed for genomic data analysis. It is composed of at least two metadata matrices, one with the phenotype metadata (i.e. clinical data) and one table mapping each data column from different assays to a entry in the phenotype

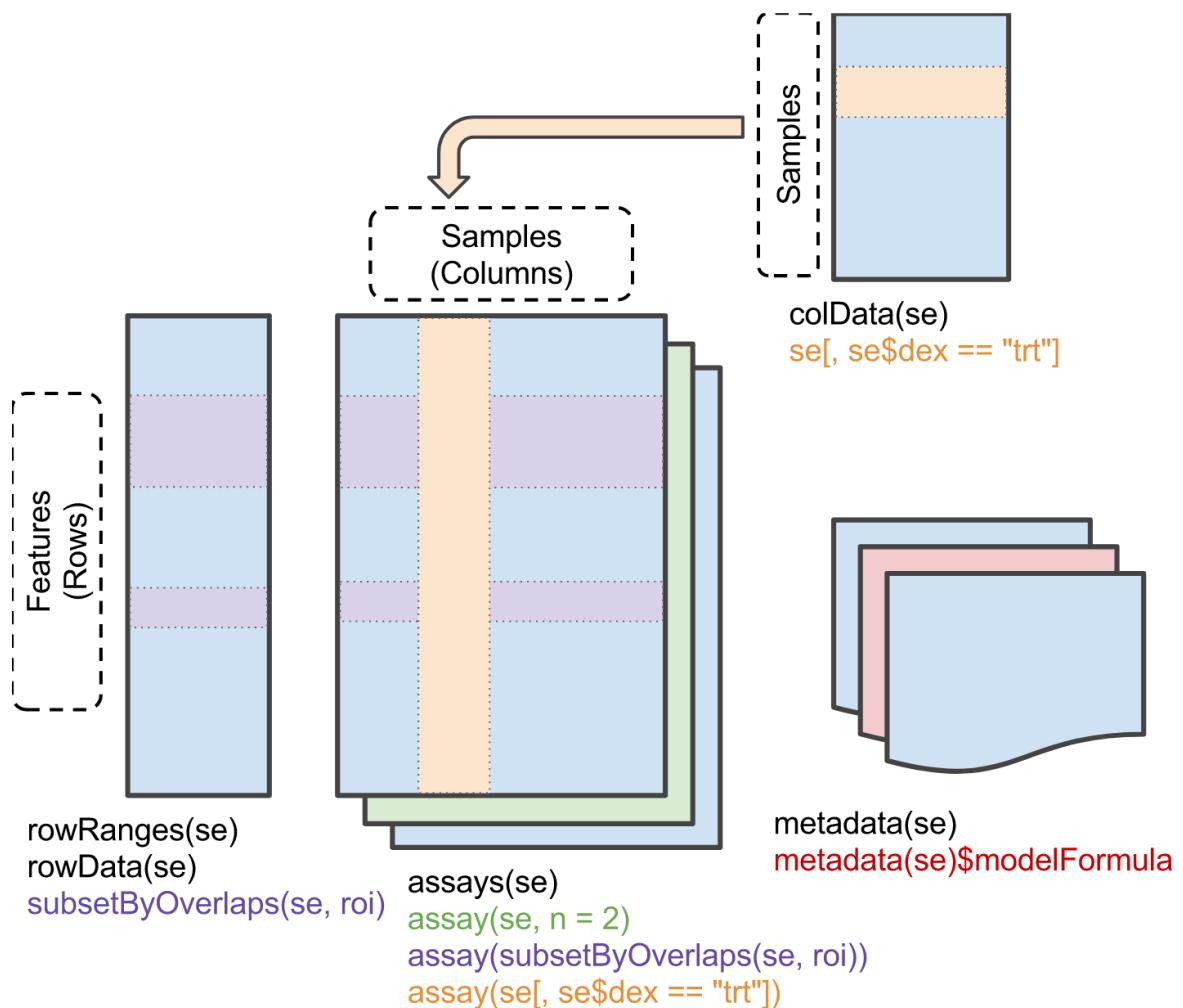


Figure 2 – Example of SummarizedExperiment object. Figure reproduced from SummarizedExperiment manual (MORGAN *et al.*, 2017).

metadata. The data handle by this data structure can be several SummarizedExperiment objects (i.e DNA methylation, gene expression, copy number, histone modification signals).

## 2.4 (Epi)Genomic data analysis

### 2.4.1 Integrative analysis

Often the onset and progression of cancerous diseases is linked to the aberrant function of proteins and alterations in gene expression, which has led research in genetics in search of the molecular alterations responsible for such aberrant behavior. Among the types of alterations genetic and epigenetic that can impact gene function are, gene copy number (CN), DNA methylation, single nucleotide variations (SNV), and indels (small insertions and deletions). These variations can have a direct affect by modifying function of the gene product, for example a indel in a coding region, or a indirect affect such as the modification of regulatory regions which can interfere with gene expression by inhibiting transcription (THINGHOLM *et*

*al.*, 2016).

Furthermore, recent technological developments have enabled the creation of genome-wide data for multiple types of variations. Although individual analyzes of these variations helped increasing knowledge of the genome and of complex disorders, integrative analyses that evaluate cancer transcriptome data in the context of other data sources are often capable of extracting deeper biological insight from the data (RHODES; CHINNAIYAN, 2005).

In this section we will highlight several integrative approaches, including meta-analysis for extracting robust profiles from independent data sets, enrichment analysis for identifying coordinately regulated functional gene modules, protein interaction networks for detecting interaction complexes deregulated in cancer, transcriptional networks for inferring regulatory mechanisms in cancer and analyses of model system profiles with human tumor profiles for inferring activity of oncogenic pathways.

#### 2.4.1.1 *Functional enrichment analysis of cancer signatures*

A gene set enrichment analysis (GSEA) or functional enrichment analysis is a method to identify classes of genes or proteins that are over-represented in a large set of genes or proteins, and may have an association with disease phenotypes. This type of analysis integrates differentially expressed genes identified by a differential expression analysis (DEA) and integrating these information with external functional information is necessary for interpreting and summarizing large cancer signatures. Most approaches use external annotation databases such as Gene Ontology which is a database of controlled vocabulary gene annotations describing the biological processes, molecular functions and cellular localizations of genes as a resource for enrichment analysis in cancer signatures.

Let  $L$  be a list of genes ranked by their differential expression between the classes, the goal of GSEA is to determine whether members of a gene set  $S$  tend to occur toward the top (or bottom) this list, which might correlated with the phenotypic class distinction (SUBRAMANIAN *et al.*, 2005).

#### 2.4.1.2 *Protein interaction networks and cancer signatures*

A major objective of systems biology is to organize molecular interactions as networks (VINAYAGAM *et al.*, 2014). Protein-protein interactions (PPIs) are essential to almost every process in a cell, and they are not only crucial for understanding cell physiology in normal and disease states since the disruption of protein-protein interactions may result in disruption of the cell component or process to which they contribute, compromising the cell viability or even leading to cell death, but also for the drug development, since drugs can affect PPIs (EMBL-EBI, 2017; ALZATE, 2009). These interaction are represented as Protein-protein interaction networks (PPIN), a mathematical representations of the physical contacts between proteins in the cell.

The totality of PPIs that happen in a cell, an organism or a specific biological context is called interactome.

A detailed human interactome network that captures the entire cellular network is invaluable in interpreting cancer signatures, allowing one to infer activated subnetworks and specific proteins that are most important to a subnetwork. For example, for a given set of overexpressed proteins in the same subnetwork, one specific protein that interacts with the entire subnetwork might be the key to control the expression the entire subnetwork.

A lot of this information is available through molecular interaction databases such as IntAct (<http://www.ebi.ac.uk/intact>) (ORCHARD *et al.*, 2013).

#### 2.4.1.3 Transcriptional targets and cancer signatures

Similar to the Protein-protein interaction networks, global transcriptional networks, which defines directional pathways (i.e. which gene actives are activated by a given gene), have the potential to improve the interpretation of cancer signatures. For example, if the targets of all transcription factors were known, then one could easily infer which transcription factors must be activated in a tumor to yield the observed cancer signature. With that information, it is possible to reduce a complex cancer signature to a small number of activated transcriptional factors which will be potential therapeutic targets.

The identification of transcription factor-binding sites (TFBSs) is done using high throughput experimental methods such as ChIP-Chip and ChIP-sequencing (ChIP-seq) which identifies a region of 100–1000 base pairs (b.p.) in which the TFBS (typically 9-15 b.p.) resides (JAYARAM; USVYAT; MARTIN, 2016). In-silico sequence-based methods were developed to predict TFBSs. These methods scan a DNA sequence of interest with a position weight matrix (PWM), a  $4 \times n$  matrix of scores for each of the 4 bases across each position in the binding motif, for a transcription factor of interest and perform a pattern-matching. PWM models can be obtained from a number of resources including the open access database JASPAR (PORTALES-CASAMAR *et al.*, 2009), HOCOMOCO (KULAKOVSKIY *et al.*, 2013) and HOMER (HEINZ *et al.*, 2010). Among the existing software that scans a sequence database for individual matches to each of the motifs are Find Individual Motif Occurrences (FIMO) (GRANT; BAILEY; NOBLE, 2011), HOMER (HEINZ *et al.*, 2010) and PATSER (TURATSINZE *et al.*, 2008).

#### 2.4.1.4 Human Epigenomes

The human body contains more than 200 different cell types each one has an identical copy of the genome but expresses a distinct set of genes, due to their epigenome which in each cell regulates gene expression in a number of ways - by organizing the nuclear architecture of the chromosomes, restricting or facilitating transcription factor access to DNA, and preserving a memory of past transcriptional activities (RIVERA; REN, 2013). The integrative analysis of

epigenomic maps, which references to the collection of DNA methylation state and covalent modification of histone proteins along the genome (BONASIO; TU; REINBERG, 2010), has been shown to be important in the study of the gene regulatory programs (GIFFORD *et al.*, 2013; HAWKINS *et al.*, 2010; RADA-IGLESIAS *et al.*, 2012). Table 3 shows a summary of the epigenomic marks and their associate role.

Table 3 – Core set of five histone modification marks and other epigenomic marks

| Histone marks                                  | Role   |
|--|--|
| Histone H3 lysine 4 trimethylation (H3K4me3)   | Promoter regions (HEINTZMAN <i>et al.</i> , 2007; BERNSTEIN <i>et al.</i> , 2005)  |
| Histone H3 lysine 4 monomethylation (H3K4me1)  | Enhancer regions (HEINTZMAN <i>et al.</i> , 2007)  |
| Histone H3 lysine 36 trimethylation (H3K36me3) | Transcribed regions  |
| Histone H3 lysine 27 trimethylation (H3K27me3) | Polycomb repression (BONASIO; TU; REINBERG, 2010)  |
| Histone H3 lysine 9 trimethylation (H3K9me3)   | Heterochromatin regions (PETERS <i>et al.</i> , 2003)  |
| Histone H3 acetylated at lysine 27 (H3K27ac)   | Increase activation of genomic elements (HEINTZMAN <i>et al.</i> , 2009; RADA-IGLESIAS <i>et al.</i> , 2011; CREYGHTON <i>et al.</i> , 2010) |
| Histone H3 lysine 9 acetylation (H3K9ac)       | Transcriptional activation (NISHIDA <i>et al.</i> , 2006)  |
| DNase hypersensitivity                         | Regions of accessible chromatin (THURMAN <i>et al.</i> , 2012)   |
| DNA methylation                                | Repressed regulatory regions (CEDAR; BERGMAN, 2009; MOORE; LE; FAN, 2013)  |

Using these set of epigenetics marks, it is possible to discover epigenomic states which would aid in understanding “non-coding” genomic elements (COETZEE *et al.*, 2017). Several tools can use those marks to infer chromatin-state such as ChromHMM (ERNST; KELLIS, 2012) and StatePaintR (COETZEE *et al.*, 2017). A 15-state model created from ROADMAP data using ChromHMM is presented as follows: active TSS-proximal promoter states (TssA, TssAFlnk), a transcribed state at the 5' and 3' end of genes showing both promoter and enhancer signatures (TxFlnk), actively-transcribed states (Tx, TxWk), enhancer states (Enh, EnhG), and a state associated with zinc finger protein genes (ZNF/Rpts). The inactive states consist of constitutive heterochromatin (Het), bivalent regulatory states (TssBiv, BivFlnk, EnhBiv), repressed Polycomb states (ReprPC, ReprPCWk), and a quiescent state (Quies) (KUNDAJE *et al.*, 2015). Those precomputed chromatin-state segmentation from public databases such as Blueprint, ENCODE and ROADMAP using ChromHMM (<http://compbio.mit.edu/ChromHMM/>) are available at [http://egg2.wustl.edu/roadmap/web\\_portal/chr\\_state\\_learning.html#core\\_15state](http://egg2.wustl.edu/roadmap/web_portal/chr_state_learning.html#core_15state) and using StatePaintR are available at [www.statehub.org](http://www.statehub.org).

These data is extremely useful to characterize each different region of the genome. For example, using those information Kundaje *et al.* (2015) were able to identify enhancer regions containing strong H3K27ac signal which showed a higher DNA accessibility, lower methylation, and higher TF binding than enhancers regions lacking H3K27ac. Those regions with strong H3K27ac signal are more likely to have a regulatory role.

## 2.4.2 Statistical analysis

### 2.4.2.1 Hypothesis Testing

Human genetic studies aims to identify if a phenotype is related to the genotypes at various loci, that is, if genetic variations have an influence on risk of disease or other health-related phenotypes. Statistical analysis is a crucial to present the findings in an interpretable and objective manner (SHAM; PURCELL, 2014).

The most popular hypothesis testing approach used to test if genotypes and phenotypes are related is the frequentist significance testing approach. This is a classical approach that involves setting up two competing hypothesis: a null hypothesis ( $H_0$ ) and an alternative hypothesis ( $H_1$ ).

Computing the statistical significance can be done using a one-tailed or a two-tailed test. A two-sided test is appropriate to evaluate both direction of the test, for example, is the estimated value smaller or higher than the reference, which actually test if the estimated value is different from the reference. A one-sided test is appropriate to evaluate only one direction of the test, for example, is the estimated value smaller than the reference. An example in genetic studies for a two-sided test would be  $H_0$  hypothesis that genotypes has no effect on the phenotypes while the  $H_1$  hypothesis is that there is an effect. Table 4 shows other examples.

Table 4 – Example of three hypothesis tests about the population mean  $\mu$ . In genetics it could be the mean level of expression of a gene.

| Type         | Null            | Alternative        |
|--------------|-----------------|--------------------|
| Right-tailed | $H_0 : \mu = 0$ | $H_1 : \mu > 0$    |
| Left-tailed  | $H_0 : \mu = 0$ | $H_1 : \mu < 0$    |
| Two-tailed   | $H_0 : \mu = 0$ | $H_1 : \mu \neq 0$ |

Table 5 – Type I and II Errors.  $\alpha = P(\text{Type I Error})$ ,  $\beta = P(\text{Type II Error})$

| Decision            | $H_0$ is True                       | $H_0$ is False                     |
|---------------------|-------------------------------------|------------------------------------|
| Do Not Reject $H_0$ | Correct Decision                    | Incorrect Decision ( $1 - \beta$ ) |
| Reject $H_0$        | Incorrect Decision ( $1 - \alpha$ ) | Correct Decision                   |

#### 2.4.2.2 Making a decision: P-value approach

The decision to reject or accept  $H_0$  is made based on the calculation of a test statistic (T) from the observed data. As the value of T depends on particular individuals in the population, repeating the study using different random samples from the population would provide of many different values for T. These set of T can be summarized as a probability distribution.

Even though, the decision made to reject or accept  $H_0$  just state that we had enough evidence to behave one way or the other. The rejection of the null hypothesis does not prove that the alternative hypothesis is true as the acceptance the null hypothesis does not prove that the null hypothesis is true. It might happen that null hypothesis was rejected when it was true, or it was not rejected when it was false. The first error in statistics is called a Type I error ("false positive"), while the second is called a Type II error ("false negative"). Table 5 shows the relations between truth/falseness of the null hypothesis and outcomes of the test.

The rate of the type I error or significance level  $\alpha$  is the probability of having a false positive. Normally, the significance level is set to 5%, implying that it is acceptable to have a 5% probability of incorrectly rejecting the null hypothesis. With the same logic, the rate of the type II error is denoted by  $\beta$ .

To make a decision whether to reject or accept the null hypothesis, the concept of probability value was introduced. Wasserstein e Lazar (2016) defined a p-value ( $p\text{-value} \in [0, 1]$ ) as the probability under a specified statistical model, constructed under a set of assumptions (normally "null hypothesis"), that a statistical summary of the data (e.g., the sample mean difference between two compared groups) would be equal to or more extreme than its observed value (WASSERSTEIN; LAZAR, 2016). That means, the smaller the p-value, the greater the statistical incompatibility of the data with the null hypothesis and greater the p-value more compatible is the data with the null hypothesis. In summary, if P-value is small (e.g. $P\text{-value} \leq \alpha$ ) then the null hypothesis is rejected, otherwise, it is not rejected.

It is important to highlight that a p-value does not measure the size of an effect or the importance of a result. It might happen that a very small effect produces smaller p-values if the sample size is big or measurement precision is high. On the other hand, a large effect might produce higher p-values if the sample size is small or measurements are imprecise.

#### 2.4.2.3 Correcting for multiple testing

When performing a set of statistical inferences simultaneously more likely erroneous inferences are to occur. For example, if 100 tests are carried out, then 5% of them (that is 5 tests) are expected to have  $P - value < 0.05$  by chance when  $H_0$  is, in fact, true for all the tests. Compared to a single test (equations 2.1a and 2.1b), the probability of having a type 1 error multiple test is given by the equations 2.1c and 2.1d (ŠIDÁK, 1967).

$$P(\text{Making an error}) = \alpha \quad (2.1\text{a})$$

$$P(\text{Not making an error}) = 1 - \alpha \quad (2.1\text{b})$$

$$P(\text{Not making an error in } m \text{ tests}) = (1 - \alpha)^m \quad (2.1\text{c})$$

$$P(\text{Making at least 1 error in } m \text{ tests}) = 1 - (1 - \alpha)^m \quad (2.1\text{d})$$

To handle this multiple statistical testing problems, some techniques to re-calculating probabilities obtained from a statistical test which was repeated multiple times have been developed to prevent the inflation of false positive rates.

Among the different approaches to control type I errors we have Family-wise error rate (FWER) which controls the probability of at least one type I error, and False discovery rate (FDR) which controls the expected proportion of Type I errors among the rejected hypotheses. Compared to FDR, controlling FWER is extremely conservative approach as the power to detect  $H_1$  gets very small.

Among the different adjustment methods to control FWER includes the Bonferroni correction in which the p-values are multiplied by the number of comparisons ( $M * P_i < \alpha$ ) and the Holm correction,  $P - adjusted_i = P_i * (M + 1 - i)$ , where  $i \in \{1, 2, \dots, n\}$  and smaller the p-value is smaller will the index  $i$  be (AICKIN; GENSLER, 1996). The Benjamini-Hochberg (BH) method to control FDR procedure will identify the largest  $k$ , such that  $P_k \leq \frac{k}{m} \alpha$ , all null hypotheses  $H_i$  for  $i \in \{1, \dots, k\}$  are rejected.

These methods make the assumption that the tests are independent tests, which often is not valid for genomics data. For dependent tests, permutation methods are often used to calculate p-values. This approach recalculates a p-value comparing the P-value calculated from the real data test with random ones, which are performed by randomly shuffling the case-control (or phenotype) labels. All  $M$  tests are recalculated on the reshuffled data set, with the smallest P value of these  $M$  tests being recorded. The procedure is repeated for many times to construct an

empirical frequency distribution of the smallest P values. This empirical adjusted P value ( $P_*$ ) is given by:

$$P_* = \frac{r+1}{n+1}$$

where  $n$  is the number of permutation carried out, and  $r$  is the number of permuted p-values smaller than P-value calculated from the real data.

For example, considering P-value = 0.1 and the permuted p-values

$$P_{perm} = \{0.001, 0.01, 0.02, 0.03, 0.05, 0.2, 0.5, 0.6, 1\}$$

the first 5 permuted p-values are smaller than the original p-value, which would give us  $r = 5$ , resulting in:

$$P_* = \frac{r+1}{n+1} = \frac{5+1}{9+1} = 0.6$$

It is important to highlight that a high number of permutations is required in order to produce reliable permuted p-value adjusted. (DAVISON; HINKLEY, 1997; NORTH; CURTIS; SHAM, 2002; NORTH; CURTIS; SHAM, 2003; SHAM; PURCELL, 2014).

#### 2.4.2.4 Nonparametric and parametric tests

Statistical procedures can be classified into two groups: Parametric and nonparametric. Parametric statistical procedures rely on assumptions about the shape of the distribution (i.e., assume a normal distribution) in the underlying population and about the form or parameters (i.e., means and standard deviations) of the assumed distribution. While nonparametric statistical procedures don't rely or rely on only a few assumptions about the shape or parameters of the population distribution from which the sample was drawn. Some of these procedures are summarized in Table 6.

The t-test, a parametric test, is the most widely used statistical test for comparing the means of two independent groups. It assumes that the data are distributed Normally, that samples from different groups are independent and that the variances between the groups are equal. The most commonly used nonparametric test in this situation is the Wilcoxon Rank Sum Test (WRST) and the closely related Mann-Whitney U-test (MWU). The WRST assumes that observations from the different groups are random samples (i.e. independent and identically distributed) from their respective populations and are mutually independent and that the observations are ordinal or continuous measurements. When there are more than two groups being compared, the nonparametric test used is Kruskal-Wallis test (KW), a generalization of the WRST. KW is the nonparametric equivalent to Analysis of variance (ANOVA).

### 2.4.3 Survival analysis

In the medical sciences, in several circumstances, it is necessary to evaluate if a treatment had a beneficial effect on the survival of patients. For this, it is measured the fraction of patients living for a certain amount of time after treatment.

Table 6 – Summary of parametric and non parametric procedures.

| Analysis Type   | Example   | Parametric                         | Nonparametric               |
|---|---|------------------------------------|-----------------------------|
| Compare means between two distinct/independent groups                 | Is the mean TP53 gene expression for control group different from the mean for treatment group?                           | Two-sample t-test                  | Wilcoxon ranksum test       |
| Compare two quantitative measurements taken from the same individual  | Was there a change in gene expression after the treatment?  | Paired t-test                      | Wilcoxon signedrank test    |
| Compare means between three or more distinct/independent groups       | For a given three groups (e.g., placebo, drug #1, drug #2), is the TP53 gene expression different among the three groups? | Analysis of variance (ANOVA)       | Kruskal-Wallis test         |
| Estimate the degree of association between two quantitative variables | Is age related to the TP53 gene expression?   | Pearson coefficient of correlation | Spearman's rank correlation |

It is called "Survival times" data that measure follow-up time from a defined starting time to the occurrence of a given event (e.g. from the diagnosis of a disease to death) (BEWICK; CHEEK; BALL, 2004). These survival times are "censored" when there is a follow-up time but the event has not yet occurred or is not known to have occurred. That might happen if a patient drops out of the study before its end, or if you are studying a treatment and it is not over yet.

#### 2.4.3.1 Kaplan-Meier method: Estimating the survival curve

It is defined survival function  $S(t)$  is defined as the probability of surviving at least to time  $t$ , while a graph of  $S(t)$  against  $t$  is called the survival curve. To estimate this curve there exists the Kaplan-Meier method:

$$S(t) = \prod_{i:t_i \leq t} \left(1 - \frac{d_i}{n_i}\right),$$

where  $d_i$  are the number of events and  $n_i$  the total individuals at risk at time  $i$ . Table 7 shows an example of survival data and Table 8 shows and example of applying Kaplan-Meier method. It is important to highlight that for a censored time the proportion surviving will be 1, that means an individual is considered to be at risk of dying in the next event of the censoring but not in subsequent events (BLAND; ALTMAN, 2004).

#### 2.4.3.2 Comparing survival curves of two groups using the log rank test

To compare the survival distributions of two or more groups, the hypothesis test log-rank test is used to test the null hypothesis that there is no difference between the populations in

Table 7 – Survival time and status for a group of patients.

| Patient ID | Survival times (in days) | Status           |
|------------|--------------------------|------------------|
| 1          | 1                        | Dead             |
| 2          | 1                        | Dead             |
| 3          | 2                        | Alive (censored) |
| 4          | 3                        | Dead             |

Table 8 – Kaplan-Meier method example for table 7

| Interval                   | $n_i$ : patient at risk at time $t_i^-$ | $d_i = \text{deaths at time } t_i$ | $c_i = \text{censored at time } t_i$ | $S(t)$                        |
|----------------------------|---|------------------------------------|--------------------------------------|-------------------------------|
| $[0, 1)$                   | 4                                       | 0                                  | 0                                    | 1                             |
| $[1, 3)$                   | $4 - 0 = 4$                             | 2                                  | 1                                    | $1 - \frac{2}{4} = 0.5$       |
| $[3, \text{End of study}]$ | $4 - 2 - 1 = 1$                         | 1                                  | 0                                    | $0.5 * (1 - \frac{1}{1}) = 0$ |

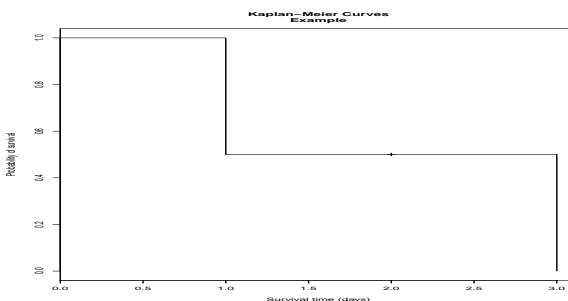


Figure 3 – Example of survival curve for table 7. Censored data is marked (+) in the plot.

the probability of an event at any time point. The approximated statistics used for comparison purposes for  $k$  groups is

$$T = \sum_{i \in \{1, \dots, k\}} \frac{(O_i - E_i)^2}{E_i},$$

where  $O_i$  is the observed numbers of death in group  $i$  while  $E_i$  is its expected numbers of deaths. If the null hypothesis is true,  $T$  is distributed approximately as a  $\chi^2_{k-1}$  (MATTHEWS; FAREWELL *et al.*, 1996). If  $T$  calculated is 9.44, and  $k = 2$ , evaluating the quantile function (also known as “inverse CDF” or “ICDF”) of the chi-squared distribution the significance level of these data is equal to  $P_r(\chi^2_1 \geq 9.44 = 0.002)$  (YAU, 2012). For a given cut-off, normally 0.05, the results with a p-value smaller are considered significant.

#### 2.4.4 Machine Learning

Machine learning is a field of computer science focused on the development and application of algorithms that improve with experience (MITCHELL *et al.*, 1997).

In genetics and genomics, it has been applied for the interpretation of large genomic data sets and annotation of a wide variety of genomic sequence elements. For example, for the detection of transcription start sites (TSSs) locations, which have proven hard to detect in silico due to the complexity and the fairly diffuse structures of Eukaryotic promoters, Down e Hubbard

(2002) developed a machine-learning method is able to build useful models of promoters for > 50% of human transcription start sites (DOWN; HUBBARD, 2002).

The machine learning techniques can be classified into two main categories: supervised and unsupervised learning (Mitchell, 1997). The supervised learning, which aims to infer data labels by learning from already labeled data, has three stages: design, model, and test. The first stage refers to the selection of a learning algorithm used to learn from data (e.g. choose between support vector machines or random forest algorithms) and its training data. The second stage is the creation of a model from labeled data using the algorithm selected previously. The last stage uses of this generated model to predict the labels of unlabeled data. The unsupervised learning methods, on the other hand, cluster the data without using labels, which requires an additional step in which semantics must be manually assigned to each cluster. As this discovery is not tied to previously defined classes, these methods have as benefit the ability to identify potentially novel types of genomic elements.

#### 2.4.4.1 *Supervised learning*

Supervised learning is a type of machine learning algorithm that identify patterns in data to make predictions. Specifically, the algorithm takes a known set of input data and known responses to the data (labels), and trains a model to generate predictions for the response to new data. Supervised learning algorithm can be classified into two categories: classification and regression.

In classification, the goal is to assign a label to an observation. That is, responses are categorical variables. For example, given dataset known to be enhancer or not enhancer, one could want to predict the locations of enhancers in the genome.

In regression, the goal is to predict a continuous measurement for an observation. That is, the responses variables are real numbers. In genomics, these methods are used in genetic epidemiology to detect association of genetic variants with a trait or disease of interest (DASGUPTA *et al.*, 2011).

#### 2.4.4.2 *Unsupervised learning*

Unsupervised learning is a type of machine learning algorithm used to draw inferences from datasets consisting of input data without labeled responses. The most common unsupervised learning method is cluster analysis, which is used for exploratory data analysis to find hidden patterns or grouping in data. These algorithms seeks to group a set of objects into cluster (groups) such that those in the same group are more similar to each other than to those in other cluster. The major unsupervised learning techniques used in bioinformatics are described below.

**Hierarchical clustering:** This clustering methods builds a hierarchy of clusters, through either a agglomerative ("bottom up") or a divisive ("top down") procedures. In the agglomerative

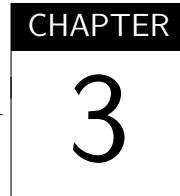
procedures, each  $n$  observation starts in its own cluster and until only one cluster remains the groups with the smallest dissimilarity are merged. On the other hand, in the divisive procedures, all observations start in one cluster and until all observations are in their own cluster the group is split in two groups with biggest dissimilarity. To decide which clusters to merge or divide a metric to measure of dissimilarity between sets of observations is required. Among the existing agglomerative techniques are the single linkage, also known as the nearest-neighbour technique, which defines the smallest dissimilarity between two points in each group as the dissimilarity between two groups (FLOREK *et al.*, 1951), the complete linkage which defines the largest dissimilarity between two points in each group as the dissimilarity between two groups (DEFAYS, 1977), the average linkage which defines the average dissimilarity over all points as the dissimilarity between two groups (SOKAL, 1958) and the Ward's method which at each merge step minimizes the increase in the total within-cluster error sum of squares, which means that the groups leading to minimum increase in total within-cluster variance are merged (JR, 1963; EVERITT *et al.*, 2011). The results of hierarchical clustering are usually represented in a dendrogram a branching diagram in which the objects are represented in one of the axis while the similarity between clusters are represented in the other axis by the length of the connection which joins them (MANNING; SCHÜTZE *et al.*, 1999).

**Optimization clustering techniques:** These are a set of non-hierarchical clustering techniques that cluster individuals into a specified number of groups, by either minimizing or maximizing some numerical criterion (EVERITT *et al.*, 2011). One of these technique is the k-means algorithms, which iteratively updates a partition by simultaneously relocating each object to the group to whose mean it was closest and then recalculating the group means until the groups no longer change. More explicitly, let  $S = \{S_1, S_2, \dots, S_k\}$  be the set of  $k$  clusters, this algorithms tries to minimize the squared distances of the elements from the cluster center:

$$N(n, k) = \min \sum_{g=1}^k \sum_{j \in S_g} \|x_j - \mu_g\|^2,$$

where  $x_j$  is a data element that belongs to a group  $g$ , and  $\mu_g$  is the center of points of the  $g$  group. A counterpoint of these techniques also requirement to 'estimate' the number of clusters in the data, for which a variety of methods have been suggested and most are relatively informal and involve, essentially, plotting the value of the clustering criterion against the number of groups in which large changes of levels in the plot are usually taken as suggestive of a particular number of groups. Some formal methods were also proposed such as the Silhouette index by Rousseeuw (1987). And a second counterpoint of this technique is the choice of initial starting values which might lead to local optimal solution, some solution to this problem such as a bootstrap-like approach to 'refine' initial seeds are suggested (STEINLEY, 2003).

**Principal component analysis (PCA):** This method transforms the variables in a multivariate data set into new variables, a linear combination of the original variables, which are uncorrelated with each other and a linear combination of the original variables. This technique provides a means of projecting the data into a lower dimensional space, which is very useful mainly due to the problems of high dimensional data which is called “curse of dimensionality”. Generally, Verleysen e François (2005) defines the curse of dimensionality as the expression of all phenomena that appear with high-dimensional data, and that have most often unfortunate consequences on the behavior and performances of learning algorithms. In summary, the number of learning data should grow exponentially with the dimension, that means if 10 data is reasonable to learn a 1-dimensional model, for a 2-dimensional model it will be need 100 data). For example, in genetics, microarray experiments have information for thousand of genes, if each one is considered a variable we would have thousand dimensions, and we would need an enormous amount of observations to obtain a reliable result. If a dimensionality reduction is not performed, with a increase of data features (dimensions), it is very likely that, by chance, one feature perfectly separates the training examples into positive and negative classes, which would lead to good performance on the training data but poor generalization to data that were not used in training (LIBBRECHT; NOBLE, 2015).



# DEVELOPMENT OF METHODS AND SOFTWARES FOR CANCER DATA ANALYSIS

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This chapter introduces three R/Bioconductor packages developed during the doctoral project. These tools are complementary and together they are able to perform a deep integrative analysis of genomic and epigenomic data from different databases, from the data acquisition stage to the integrative analysis.

First, to deal with the problem of data acquisition and analysis we created the R/Bioconductor TCGAbiolinks package which searches, downloads and organizes data and metadata from the National Cancer Institute's (NCI) Genomic Data Commons (GDC). Second, to deal with the problem of integrative analysis, we worked closely with Dr. Benjamin P. Berman group to create a new version of the R/Bioconductor ELMER package, which infers regulatory element landscapes and transcription factor networks from cancer methylomes (YAO *et al.*, 2015). This version not only was greatly improved in terms of stability, performance, and extensibility, but it also added a number of new features. Finally, to improve the usability of the tools developed, we used the R web application framework shiny (CHANG *et al.*, 2016) to create the R/Bioconductor TCGAbiolinksGUI package, which provides a graphical interface to ours and several Bioconductor packages.

## 3.1 TCGAbiolinks: An R/Bioconductor package to download and analyze data from GDC

The aim of TCGAbiolinks is four-fold: (i) to facilitate data retrieval via GDC's API; (ii) to prepare the data using the appropriate preprocessing strategies; (iii) to provide a means to conduct different standard analyses and advanced integrative analyses and (iv) to allow the user to easily reproduce earlier research results. We introduce public methods used in several marker papers to integrate DNA methylation and gene expression data. In addition, our tool extracts published molecular subtype information for each TCGA sample within a tumor type (generally embedded

in supplementary tables, PDFs or external websites). Because our tool was developed in the language of R specifically for integration within the Bioconductor project, we have provided most of the TCGA data objects as the Bioconductor specified ‘SummarizedExperiment’ class (HUBER *et al.*, 2015), thereby allowing easy integration with other data types and statistical methods that are common in the Bioconductor repository.

### 3.1.1 The *TCGAbiolinks* package

TCGAbiolinks is an R package, which is licensed under the General Public License (GPLv3), and is freely available through the Bioconductor repository (GENTLEMAN *et al.*, 2004). By conforming to the strict guidelines for package submission to Bioconductor, we were able to utilize and incorporate existing R/Bioconductor packages and statistics to assist in identifying differentially altered genomic regions defined by mutation, copy number, expression or DNA methylation; to reproduce previous TCGA marker studies; and to integrate data types both within TCGA and across other data types outside of TCGA. TCGAbiolinks consists of functions that can be grouped into three main levels: Data, Analysis and Visualization. More specifically, the package provides multiple methods for the analysis of individual experimental platforms (e.g. differential expression analysis or identifying differentially methylated regions or copy number alterations) and methods for visualization (e.g. survival plots, volcano plots and starburst plots) to facilitate the development of complete analysis pipelines. In addition, TCGAbiolinks offers in-depth integrative analysis of multiple platforms, such as copy number and expression or expression and DNA methylation, as demonstrated and applied in our recent TCGA study of 1122 gliomas (CECCARELLI *et al.*, 2016b). These functions can be used independently or in combination to provide the user with fully comprehensible analysis pipelines applied to TCGA data. A schematic overview of the package is presented in Figure 4. The next subsections describe each of the three main levels (Data, Analysis and Visualization) below, highlighting the importance and utility of each associated function and sub-function.

#### *Data*

TCGA data is accessible via the [NCI Genomic Data Commons \(GDC\) data portal](#), [GDC Legacy Archive](#) and [the Broad Institute’s GDAC Firehose](#). The GDC Data Portal provides access to the subset of TCGA data that has been harmonized against Genome Reference Consortium Human Build 38 (GRCh38) (hg38) using GDC Bioinformatics Pipelines which provides methods to the standardization of biospecimen and clinical data, the re-alignment of DNA and RNA sequence data against a common reference genome build GRCh38, and the generation of derived data. Whereas the GDC Legacy Archive provides access to an unmodified copy of data that was previously stored in CGHub (WILKS *et al.*, 2014) and in the TCGA Data Portal hosted by the TCGA Data Coordinating Center (DCC), in which uses as references Genome Reference Consortium Human Build 37 (GRCh37) (hg19) and Genome Reference Consortium Human

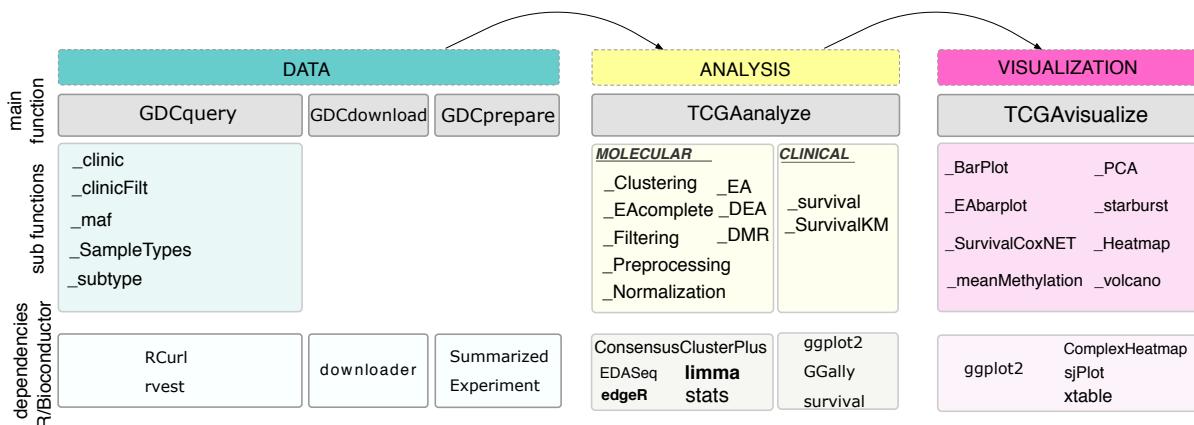


Figure 4 – Overview of TCGAbiolinks functions. TCGAbiolinks is organized in three categories. In the first category (Data), functions to query the GDC database, to download the data and to prepare it are made available. The second category (Analysis) contains functions that allow the user to carry out different types of analyses; these include clustering (*TCGAanalyze\_Clustering*), differential expression analysis (*TCGAanalyze\_DEA*) and enrichment analysis (*TCGAanalyze\_EA*). Finally, the obtained results can be visualized using the functions in the third category (Visualization): these include principal component analysis (*TCGAvizualize\_PCA*), starburst plots (*TCGAvizualize\_starburst*) and survival curves (*TCGAvizualize\_SurvivalCoxNET*). The different dependencies to other R/Bioconductor packages are specified in the last row of the figure.

Build 36 (GRCh36) (hg18).

The previously stored data in CGHub, TCGA Data Portal and Broad Institute's GDAC Firehose, were provided as different levels or tiers that were defined in terms of a specific combination of both processing level (raw, normalized, integrated) and access level (controlled or open access). Level 1 indicated raw and controlled data, level 2 indicated processed and controlled data, level 3 indicated Segmented or Interpreted Data and open access and level 4 indicated region of interest and open access data. While the TCGA data portal provided level 1 to 3 data, Firehose only provides level 3 and 4. An explanation of the different levels can be found at TCGA Wiki (<https://wiki.nci.nih.gov/display/TCGA/Data+level>). However, the GDC data portal no longer uses this based classification model in levels. Instead a new data model was created, its documentation can be found in GDC documentation at <https://gdc.nci.nih.gov/developers/gdc-data-model/gdc-data-model-components>. In this new model, data can be open or controlled access. While the GDC open access data does not require authentication or authorization to access it and generally includes high level genomic data that is not individually identifiable, as well as most clinical and all biospecimen data elements, the GDC controlled access data requires dbGaP authorization and eRA Commons authentication and generally includes individually identifiable data such as low level genomic sequencing data, germline variants, SNP6 genotype data, and certain clinical data elements. The process to obtain access to controlled data is found in GDC web site at <https://gdc.nci.nih.gov/access-data/obtaining-access-controlled-data>.

TCGAbiolinks divides the GDC data retrieval into three main functions: *GDCquery*, *GDCdownload* and *GDCprepare*. *GDCquery* allows the user to query data from the NCI's Genomic Data Commons (GDC) data portal or GDC Legacy Archive by accessing the

database API. Up to the moment, the GDC data portal provides data from two programs The Cancer Genome Atlas (TCGA) and Therapeutically Applicable Research to Generate Effective Treatments (TARGET) on more than 38 diseases type cancer types and 5 different molecular data types (Transcriptome Profiling, Raw Sequencing Data, Copy Number Variation, DNA Methylation) as well as 3 different types of clinical reports (clinical tables, pathology reports and histology image slides). The pathology reports and histology image slides are not prepared but are downloaded from the GDC Legacy Archive to a directory if requested by the user. *GDCdownload* receives the output of *GDCquery*, a file manifest with all the metadata necessary to download it. In order to organize the files downloaded, this function will save the data with the following pattern "Root directory/project/source/data\_category/data\_type/file\_id/file\_name" (i.e Example: GDCdata/TCGA-GBM/harmonized/DNA\_Methylation/Methylation\_Beta\_Value/079fcaff-3ae6-4150-b2e6-2b7330ffbcd9/jhu-usc.edu\_GBM.HumanMethylation450.10.lvl-3.TCGA-19-A6J5-01A-21D-A33U-05.gdc\_hg38.txt) If a file was previously downloaded it will not be re-downloaded. *TCGAPrepare* is a function that reads open processed data and prepares them for downstream analysis. Specifically, the objects are organized in a *SummarizedExperiment* object to allow easy integration with other Bioconductor packages, such as GRanges (LAWRENCE *et al.*, 2013), IRanges (LAWRENCE *et al.*, 2013), limma (RITCHIE *et al.*, 2015) and edgeR (ROBINSON; MCCARTHY; SMYTH, 2010). The samples are always referred to by their given TCGA/TARGET barcode. If the user prefers the data not to be prepared in a *SummarizedExperiment*, there is an option to set the argument *SummarizedExperiment* to FALSE; the data are then prepared as a standard data frame object (rows and columns).

## Analysis

The analysis functions and subfunctions are designed to analyze TCGA data through both common and novel methods. The main function, called *TCGAanalyze*, comprises two distinct types of analysis: molecular analysis and clinical analysis. Once the data are prepared into data matrices (genes/loci in rows and samples in columns) or a *SummarizedExperiment*, the downstream analysis can be divided into (i) supervised analysis: differential expression analysis, enrichment analysis and master regulator analysis or (ii) unsupervised analysis: inference of gene regulatory network, cluster, classification, Receiver Operator Characteristics (ROC) (SONEGO; KOCSOR; PONGOR, 2008), Area Under the Curve (AUC), feature selection and survival analysis. *TCGAanalyze\_Normalization* allows users to normalize mRNA transcripts and miRNA using the EDASeq package (RISSO *et al.*, 2011). This function uses within-lane normalization procedures to adjust for GC-content effects (or other gene-level effects) on read counts: LOESS robust local regression and global-scaling, full-quantile and between-lane normalization procedures to adjust for distributional differences between lanes (e.g. sequencing depth). *TCGAanalyze DEA* allows the user to identify differential expression or regions between two populations or conditions. In particular, we used the edgeR package from Bioconductor, which uses the quantile-adjusted conditional maximum likelihood (qCML) method for experiments

with a single factor to detect differentially expressed genes (DEGs) (ROBINSON; MCCARTHY; SMYTH, 2010). Compared to several other estimators, qCML is the most reliable in terms of bias on a wide range of conditions; specifically, qCML performs best in situations involving many small samples with a common dispersion (ROBINSON; SMYTH, 2007). The P-values generated from the analysis are sorted in ascending order and corrected using the Benjamini & Hochberg procedure for multiple testing correction (BENJAMINI; HOCHBERG, 1995a). After running *TCGAanalyze\_DEA*, it is possible to filter the output by fold change and/or significance and to use the *TCGAanalyze\_LevelTab* function to create a table of DEGs, including fold change (FC), false discovery rate (FDR), gene expression levels of samples under conditions of interest and delta values (the difference in gene expression multiplied by logFC). *TCGAanalyze\_DMR* allows the user to identify differentially methylated regions (DMRs) between two groups with a DNA methylation difference above a certain threshold. To calculate P-values, this subfunction uses the Wilcoxon ranksum statistical non-parametric test and adjusts the values using the FDR method. *TCGAanalyze\_Clustering* allows the user to perform a hierarchical cluster analysis through two methods: ward.D2 and ConsensusClusterPlus (WILKERSON; HAYES, 2010).

### *Visualization*

The visualization section allows the user to visualize the results generated by the analysis sections using heatmap, cluster, plots with incremental layers (ggplot2), pathway enrichment analysis and PCA. Furthermore, we provide methods to generate a starburst plot, which integrates gene expression and DNA methylation data (NOUSHMEHR *et al.*, 2010).

### **3.1.2 Comparisons**

Recently, several tools to retrieve TCGA data sets have been made available, as summarized in Table 9. These tools include TCGA-Assembler (ZHU; QIU; JI, 2014), CGDS-R (GAO *et al.*, 2013), canEnvolve (SAMUR *et al.*, 2013), Firehose (DENG *et al.*, 2017), RTCGAToolbox (SAMUR, 2014), and cBioPortal (CERAMI *et al.*, 2012). These tools can be divided into three representative categories. The first category comprises tools mainly used to download cancer genomics data, such as TCGA-Assembler and CGDSR. The second category includes tools that focus mainly on data analysis and integration, such as canEnvolve. The third category comprises tools to download and analyze data, such as RTCGAToolbox, Firehose and cBioPortal.

RTCGAToolbox is a tool that systematically accesses the Broad GDAC Firehose (<https://gdac.broadinstitute.org/>) preprocessed data and performs basic analysis and visualization of an individual data type (expression, mutation or DNA methylation). Despite the existence of TCGA specific software packages, none of these tools perform the integrative analysis harnessing methodologies designed by TCGA Analysis working groups (AWGs), such as identifying epigenetically silenced genes (represented in a starburst plot (NOUSHMEHR *et al.*, 2010) or functional copy number identification (CECCARELLI *et al.*, 2016b). Although RTCGAToolbox

Table 9 – Each column represents a software tool compared with TCGAbiolinks, and each row represents a feature. The cells checked with X indicates features that exists in the tool. Available platform abbreviations are defined as: R (R script); C (R package deposited in CRAN); B (Bioconductor package); W (available only as a web portal);

|                      |  | Packages     |               |            |           |                       |              |                    |  |
|----------------------|--|--------------|---------------|------------|-----------|-----------------------|--------------|--------------------|--|
| Features             |  | Sub-features |               |            |           |                       |              |                    |  |
|                      |  | TCGAbiolinks | TCGAAssembler | canEnvolve | TCGA2stat | Firehose-FirebrowserR | RTCGAtoolbox | cBio Portal CGDS-R |  |
| Availability         | Platform                                       | B            | R             | W          | C         | CW                    | B            | CW                 |  |
| Genome of reference  | Access to data aligned against the GRCh38/hg38 | X            | X             |            |           |                       |              |                    |  |
|                      | Access to data aligned against the GRCh37/hg19 | X            | X             | X          | X         | X                     | X            | X                  |  |
| Query TCGA Cases     | Individual TCGA samples (e.g. TCGA-01-0001)    | X            | X             |            |           | X                     |              |                    |  |
| Download             | All TCGA platforms                             | X            |               |            |           |                       |              |                    |  |
| Data type analysis   | mRNA   | X            |               | X          | X         | X                     | X            | X                  |  |
|                      | miRNA  | X            |               | X          | X         | X                     | X            | X                  |  |
|                      | copy number                                    | X            |               | X          | X         | X                     | X            | X                  |  |
|                      | DNA methylation                                | X            |               |            | X         | X                     | X            | X                  |  |
|                      | Clinical                                       | X            |               | X          | X         | X                     | X            | X                  |  |
|                      | Protein  |              |               | X          |           | X                     |              | X                  |  |
|                      | Mutatation                                     | X            |               | X          | X         | X                     | X            | X                  |  |
| Integrative analysis | DNA methylation and gene expression            | X            |               |            |           | X                     |              |                    |  |
| Other                | Extensible to other BioC packages              | X            |               |            |           |                       |              |                    |  |

can download and analyze Firehose-generated data, neither tool can provide the downloaded data as a "SummarizedExperiment" object, which is critical for allowing the full integration and use of other popular Bioconductor packages, an integral aspect of Bioconductor (GENTLEMAN *et al.*, 2004, 2004). Briefly, the SummarizedExperiment class is a matrix-like container in which rows represent ranges of interest (as a GRanges or GRangesList object) and columns represent samples (with sample data summarized as a DataFrame). A Summarized Experiment contains one or more assays, each represented by a matrix-like object of numeric or other mode. Finally, TCGAbiolinks is able to access data aligned against the Genome Reference Consortium Human Build 38 (hg38) via the [NCI Genomic Data Commons \(GDC\) data portal](#), and data aligned against the Genome Reference Consortium Human Build 19 (hg19) via the [GDC Legacy Archive](#). This feature is only available using TCGA-Assembler.

### 3.1.3 Software availability

TCGAbiolinks is available under the GNU General Public License version 3 (GNU GPL3). Its source code is available at <https://github.com/BioinformaticsFMRP/TCGAbiolinks> and a binary version for windows, macOSX and Linux is freely available through the Bioconductor repository at <http://bioconductor.org/packages/TCGAbiolinks/>. To execute this tool, it is required to have installed a R version  $\geq 3.3$ .

### 3.1.4 Public reception

TCGAbiolinks had a good reception the research community being among the top 5% of the most downloaded tools of the Bioconductor project. In October 2017, the tool already had more than 17 thousand downloads, with an average of visits to the documentation pages of a thousand users per month. The Figure 5 shows a summary since the beginning of the project.

### 3.1.5 Case of studies

In this subsection, we introduce and describe the utility and application of TCGAbiolinks, we used four different TCGA cancer types (Brain, Kidney, Breast and Colon) as examples. For each tumor type, we describe methods to extract the different experimental types and integrate the information into a cohesive, biologically specific and hypothesis-driven approach. We also describe how to generate a starburst plot (NOUSHMEHR *et al.*, 2010). The starburst plot was introduced to illustrate the results of integrating DNA methylation and gene expression data. In addition, we describe how TCGAbiolinks prepares data for integration with other recently published packages, such as ELMER (YAO *et al.*, 2015), a new Bioconductor package designed to identify candidate regulatory elements in the non-coding regions of the genome associated with cancer. Our package is freely available within the Bioconductor project at <http://bioconductor.org/packages/TCGAbiolinks/>.

#### Case study - LGG downstream analysis with gene expression

For this case study, we used the recently available lower-grade glioma (LGG) data to investigate gene expression differences between the reported molecular subtypes (IDHmutant, IDHwildtype and IDHmutant codels) (PLATFORMS, 2015). In particular, we used TCGAbiolinks to download 293 samples profiled using messenger RNA expression (IlluminaHiSeq RNASeqV2) with available molecular subtypes. The data was normalized using the *TCGA-analyze\_Normalization* function and we applied three filters to remove features/mRNAs with low signals across samples, obtaining 4578, 4284 and 1187 mRNAs, respectively. A clustering analysis was then applied using the ConsensusClusterPlus package (WILKERSON; HAYES, 2010) which identified four distinct groups of samples (EC1-EC4) (Figure 6A). The survival curves for each cluster were generated using *TCGAanalyze\_survival* and are shown in Figure 6B.

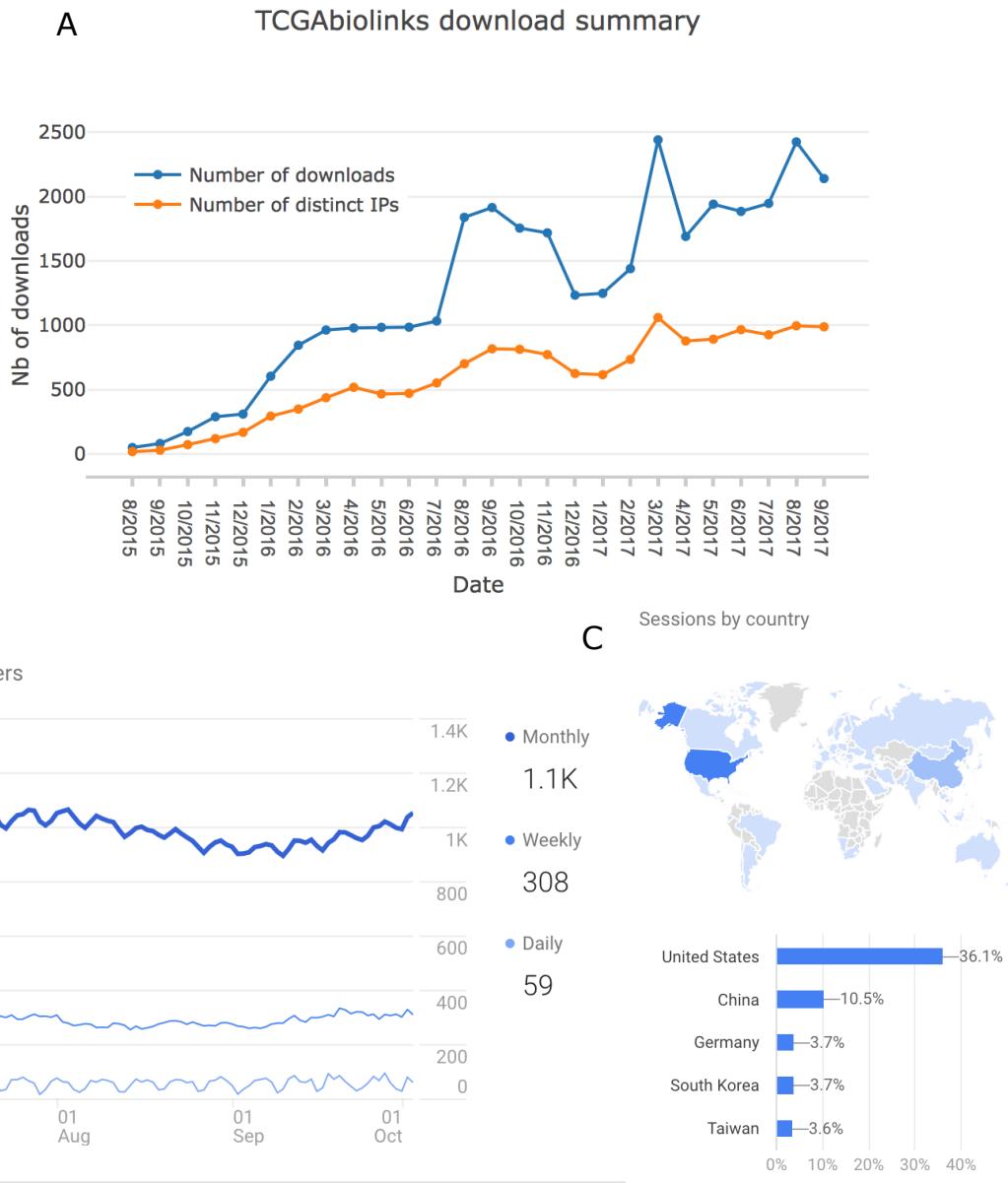


Figure 5 – TCGAbiolinks download summary. A) Number of downloads per month. B) Number of active users. C) Percentage of sessions connect to the documentation by country.

As expected, each cluster effectively separated IDHwildtype tumors (EC1) from IDHmutant-non-codel (EC2) and IDHmutant-codel tumors (EC3 and EC4) (Figure 6). Additional biological subtypes (DNA methylation subtypes) were reproduced as expected (Figure 6D) (PLATFORMS, 2015).

#### *Case study - Downstream analysis integration of gene expression and methylation data*

The DNA methylation of specific promoter CpG islands has the potential to influence gene expression. In this case study, we used TCGAbiolinks to examine the biological relationship between DNA methylation and gene expression in Colon adenocarcinoma (COAD). Using

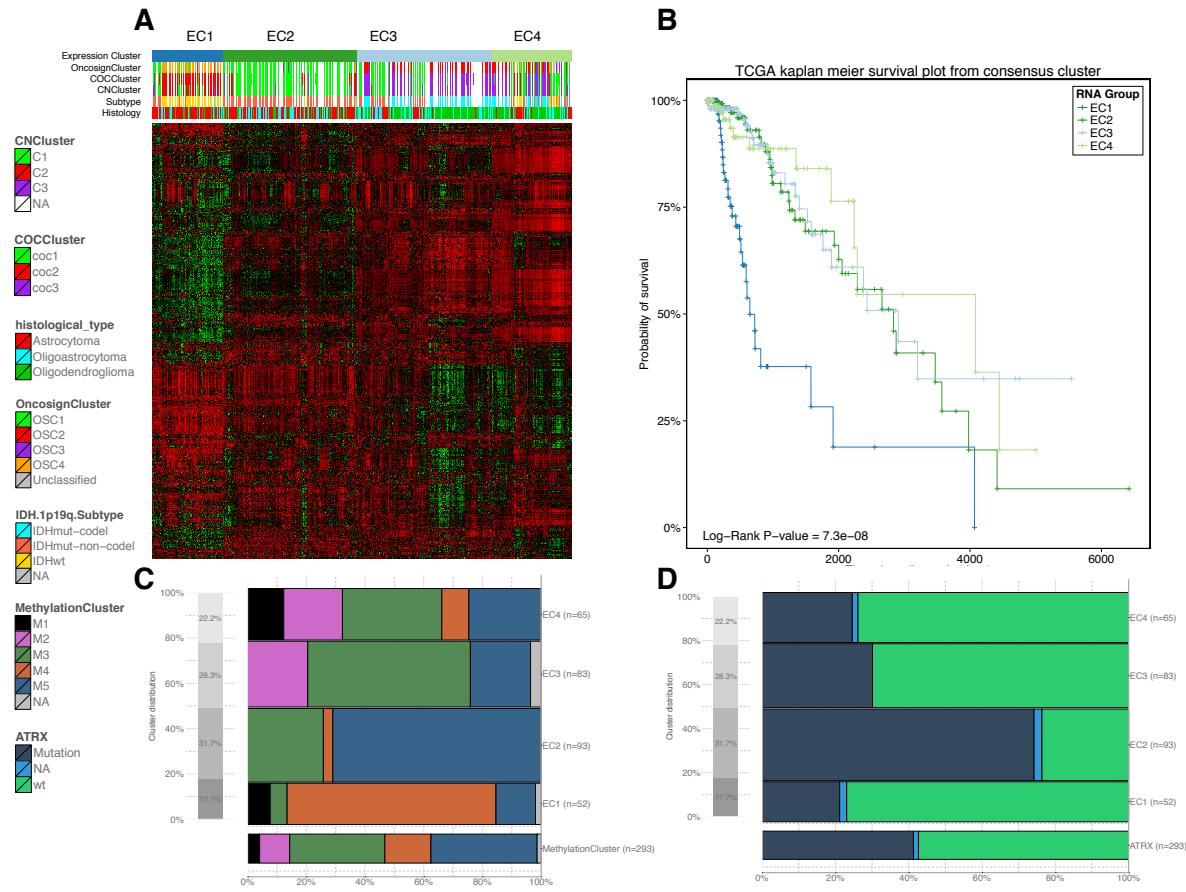


Figure 6 – Case study - Integrative (or Downstream) analysis of gene expression and clinical data from LGG disease with unsupervised clustering and crossing expression clusters with clinical and molecular information. **(A)** Heatmap of 1187 more variables genes clustered with tree  $k = 4$  in EC1, EC2, EC3, EC4. **(B)** Kaplan Meier survivals plot for EC clusters. **(C and D)** Distribution of the DNA Methylation clusters and ATRX mutation within the EC clusters.

*GDCquery*, *GDCdownload* and *GDCprepare*, we obtained DNA methylation data (Infinium HumanMethylation450 and Infinium HumanMethylation27 platforms) and gene expression data (IlluminaGA RNASeqV2 platform) for the same TCGA COAD samples (NETWORK *et al.*, 2012). A supervised analysis was performed on the molecular subtypes CIMP-Low [CIMP.L] and CIMP-High [CIMP.H]. The gene expression analysis started by the identification of outliers, followed by the normalization methods. Using *TCGAanalyze\_DEA*, 34 DEGs ( $\log_2FC \geq 3.0$  and  $FDR \leq 10^{-4}$ ) were identified. The result of this analysis is represented in a volcano plot (Figure 7A) created using *TCGAVisualize\_volcano*. For the DNA methylation analysis, using *TCGAanalyze\_DMR* we identified 73 CpG-methylated probes ( $\Delta\bar{\beta} \geq 0.25$  and  $FDR \leq 10^{-5}$ ; (Figure 7B). The DNA methylation and gene expression results were integrated as in the previous TCGA marker paper (NOUSHMEHR *et al.*, 2010; NETWORK *et al.*, 2012), by generating a starburst plot (Figure 7C) in which the x-axis is the  $\log_{10}$  of the correct P-value for DNA methylation and the y-axis is the  $\log_{10}$  of the correct P-value for the expression data. The starburst plot highlights nine distinct quadrants. To incorporate the DNA methylation difference cut-off into the graph, we highlighted genes that might have the potential for silencing due to

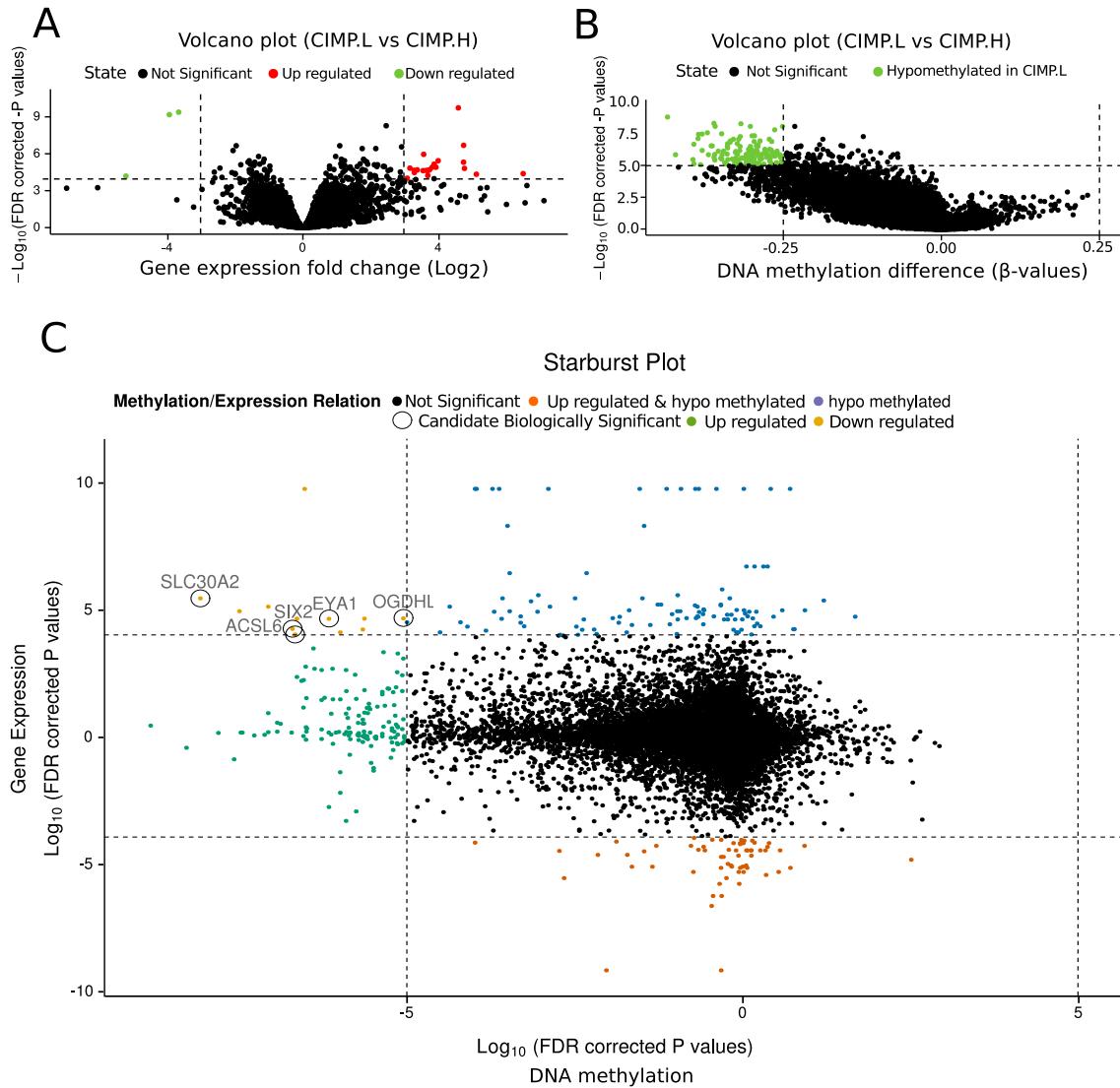


Figure 7 – Case study - Integrative analysis of gene expression and DNA methylation data from COAD disease, comparing groups CIMP.L and CIMP.H. **(A)** Expression volcano plot: fold change of expression data versus significance. **(B)** DNA methylation volcano plot: difference of DNA methylation versus significance. **(C)** Starburst plot: DNA methylation significance versus gene expression significance.

epigenetic alterations. We highlighted five genes, EYA1, SIX2, ACSL6, OGDHL and SLC30A2, that showed a  $\Delta\bar{\beta} \geq 0.25$  and a  $\log_2 FC \geq 3.0$  between CIMP.L and CIMP.H.

## 3.2 TCGAbiolinksGUI: A graphical user interface to analyze GDC cancer molecular and clinical data

Although TCGAbiolinks is a suitable R package for most data analysts with a strong knowledge and familiarity with R specifically those who can comfortably write strings of common R commands, we developed TCGAbiolinksGUI to enable user access to the methodologies offered in TCGAbiolinks and to give users the flexibility of point-and-click style analysis without the need to enter specific arguments. TCGAbiolinksGUI takes in all the important features of TCGAbiolinks and offers a graphics user interface (GUI) thereby eliminating any need to familiarize TCGAbiolinks' key functions and arguments.

### 3.2.1 *Infrastructure*

The TCGAbiolinksGUI user interface was created using Shiny, a Web Application Framework for R, and uses several packages to provide advanced features that can enhance Shiny apps, such as shinyjs to add JavaScript actions (ATTALI, 2017), shinydashboard to add dashboards (CHANG; Borges Ribeiro, 2017) and shinyFiles (PEDERSEN, 2016) to provide access to the server file system.

The following R/Bioconductor packages are used as back-ends for the data retrieval and analysis: TCGAbiolinks (COLAPRICO *et al.*, 2016) which allows to search, download and prepare data from the NCI's Genomic Data Commons (GDC) data portal into an R object and perform several downstream analysis; ELMER (Enhancer Linking by Methylation/Expression Relationship) (YAO *et al.*, 2015; SILVA *et al.*, 2017a) which identifies DNA methylation changes in distal regulatory regions and correlate these signatures with the expression of nearby genes to identify transcriptional targets associated with cancer; ComplexHeatmap (GU; EILS; SCHLESNER, 2016) to visualize data as oncoprint and heatmaps, pathview (LUO; BROUWER, 2013) which offers pathway based data integration and visualization; and maftools (MAYAKONDA; KOEFFLER, 2016) to analyze, visualize and summarize Mutation Annotation Format (MAF) files.

### 3.2.2 *Graphical user interface design*

The user interface has been divided into three main Graphical User Interface (GUI) menus. The first menu defines the acquisition of GDC data. The second defines the analysis steps which subdivides according to the molecular data types. And the third is dedicated to harnessing integrative analyses. We present below a brief description of each menu and their features that can be accessed through a side panel (see figure 8):

- **GDC Data:** Provides a guided approach to search for published molecular subtype information, clinical and molecular data. In addition, it downloads and processes the molecular

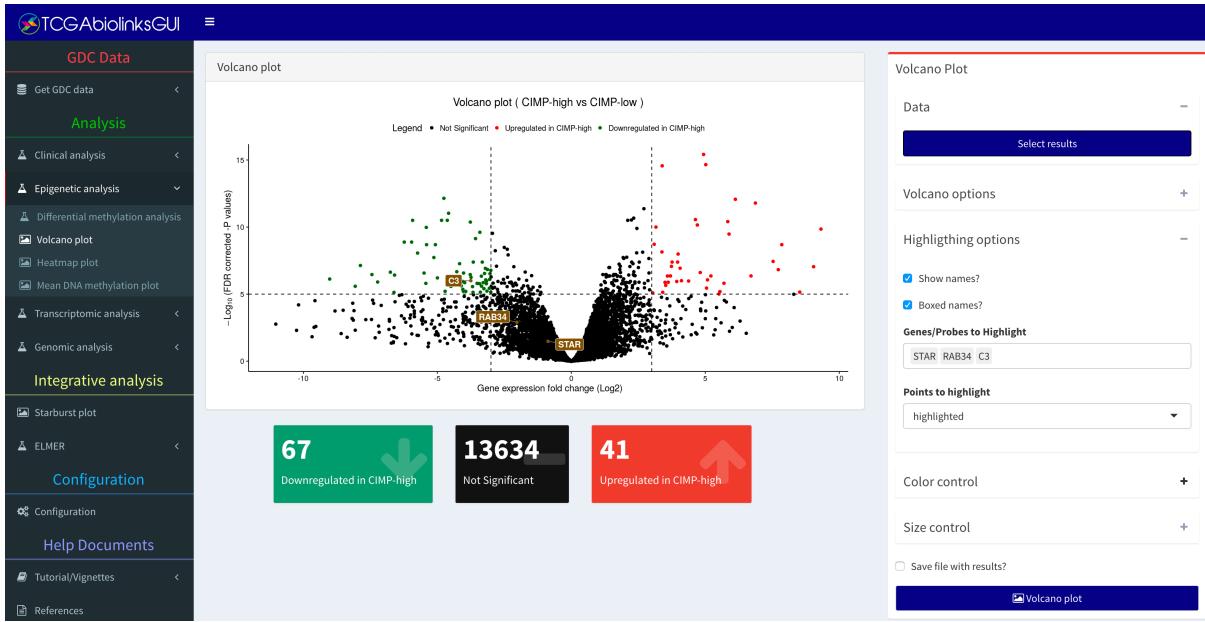


Figure 8 – The volcano plot menu of TCGAbiolinksGUI: The panel on the left shows the menus divided by different analyses, the panel on the right shows the controls available for the menu selected. In the center is a volcano plot window from the analysis menu. It is possible to control the colors, to change cut-offs and to export results into a CSV document.

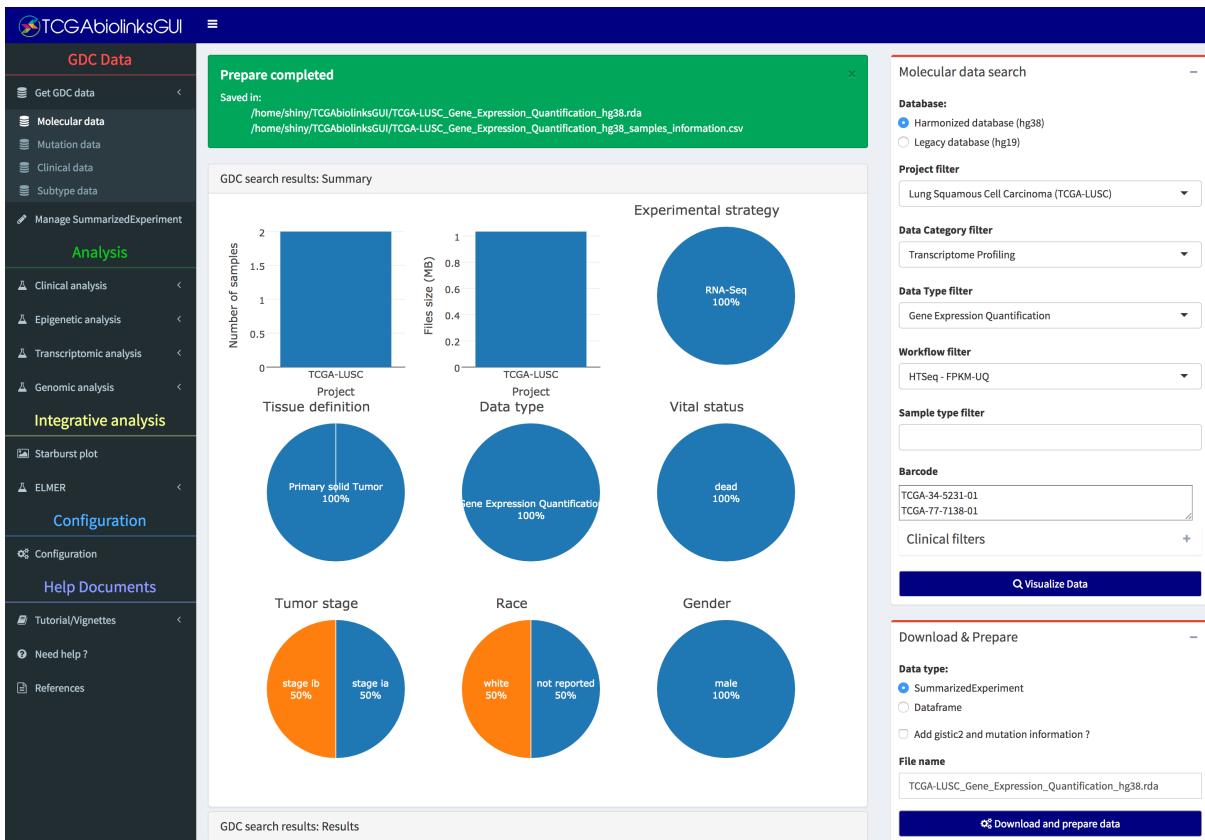


Figure 9 – Molecular data download: Gene expression Search, download and prepare into an R object of gene expression data for two TCGA-LUSC samples ("TCGA-34-5231-01", "TCGA-77-7138-01"). The Summarized Experiment object created is saved as an RData file (TCGA-LUSC-Gene\_Expression\_Quantification\_hg38.rda).

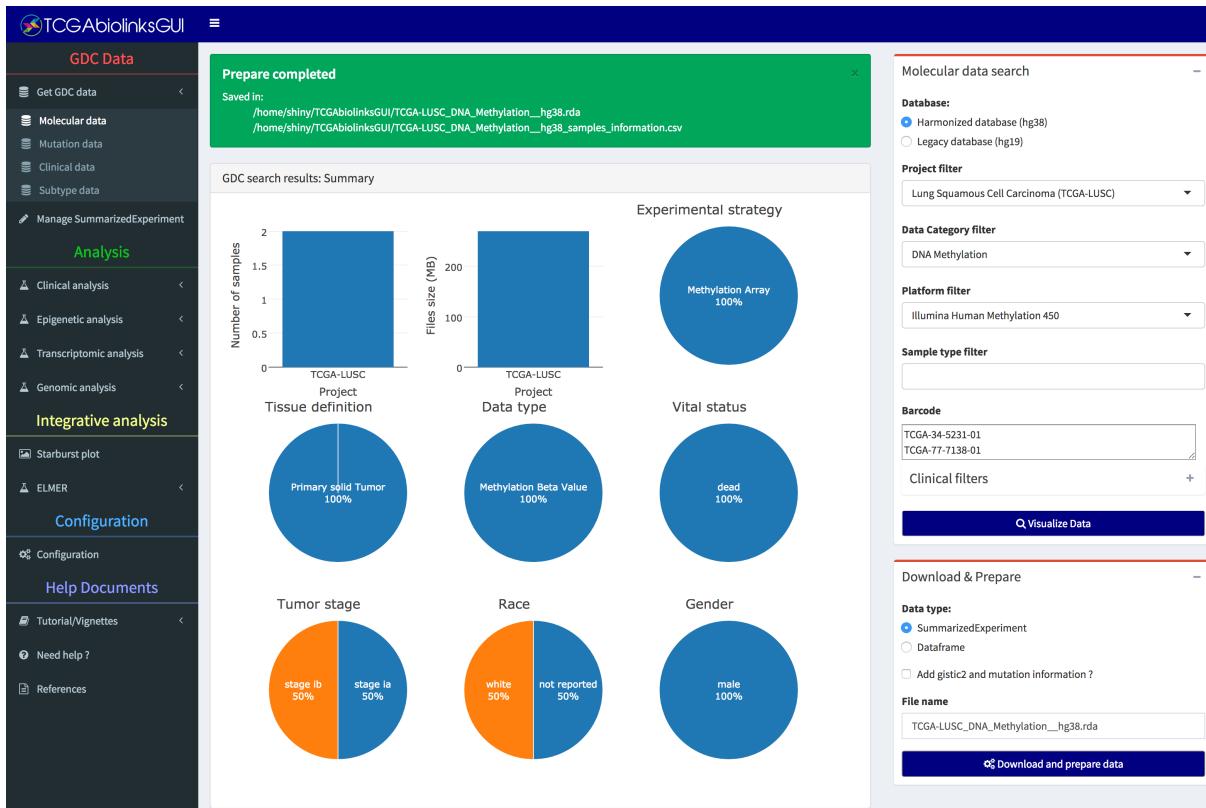


Figure 10 – Molecular data download: DNA methylation search, download and prepare into an R object of DNA methylation for two TCGA-LUSC samples ("TCGA-34-5231-01", "TCGA-77-7138-01"). The Summarized Experiment object created is saved as an RData file (TCGA-LUSC-DNA\_methylation\_hg39.rda).

data into an R object that can be used for further analysis.

- **Clinical analysis:** Performs survival analysis to quantify and test survival differences between two or more groups of patients and draws survival curves with the 'number at risk' table, the cumulative number of events table and the cumulative number of censored subjects table using the R/CRAN package *survminer* (KASSAMBARA; KOSINSKI, 2017).
- **Epigenetic analysis:** Performs a Differentially methylated regions (DMR) analysis, visualizes the results through both volcano and heatmap plots, and visualizes the mean DNA methylation level by groups.
- **Transcriptomic analysis:** Performs a Differential Expression Analysis (DEA), and visualizes the results through both volcano and heatmap plots. For the genes found as upregulated or downregulated an enrichment analysis can be performed and pathway data can be integrated (LUO; BROUWER, 2013).
- **Genomic analysis:** Visualize and summarize the mutations from MAF (Mutation Annotation Format) files through summary plots and oncoplots using the R/Bioconductor *maftools* package (GU; EILS; SCHLESNER, 2016; MAYAKONDA; KOEFFLER, 2016).

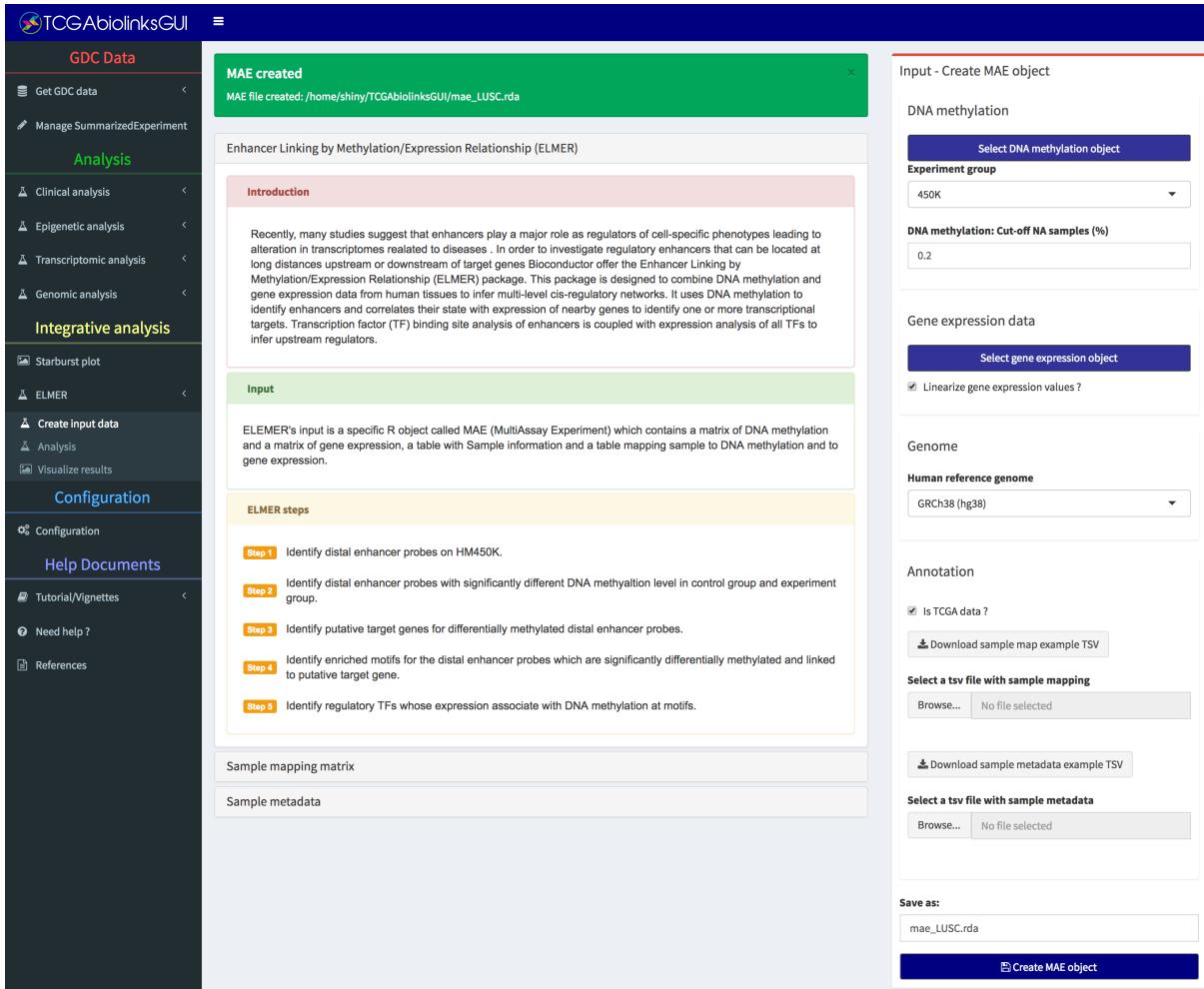


Figure 11 – ELMER input data menu: Creating a MAE (MultiAssayExperiment) object only with samples that have both DNA methylation and gene expression data. Probes will be filtered to remove those having NA (empty values) for more than 20% of the samples and only those in distal regions ( $\pm 2Kb$  away from TSS) will be kept.

- **Integrative analysis:** Integrate the DMR and DEA results through a starburst plot. Also, using the DNA methylation data and the gene expression data the R/Bioconductor ELMER package can be used to discover functionally relevant genomic regions associated with cancer (YAO *et al.*, 2015; SILVA *et al.*, 2017a).

### 3.2.3 Documentation

We provide a guided tutorial for users via a vignette document which details each step and menu function available at <http://bit.do/TCGAbiolinksDocs>, via online documents available at [http://bit.ly/TCGAbiolinks\\_PDFTutorials](http://bit.ly/TCGAbiolinks_PDFTutorials), and via YouTube video instructions showing step by step how each menu works available at [http://bit.ly/TCGAbiolinksGUI\\_videoTutorials](http://bit.ly/TCGAbiolinksGUI_videoTutorials), which assist end-users in taking full advantage of TCGAbiolinksGUI. A demonstration version of the tool is available at <http://tcgabiolinks.fmrp.usp.br:3838/>. Users are encouraged to report and file bug reports or feature requests via our GitHub repository [BioinformaticsFMRP/TCGAbiolinks-GUI/issues](https://github.com/BioinformaticsFMRP/TCGAbiolinks-GUI/issues).

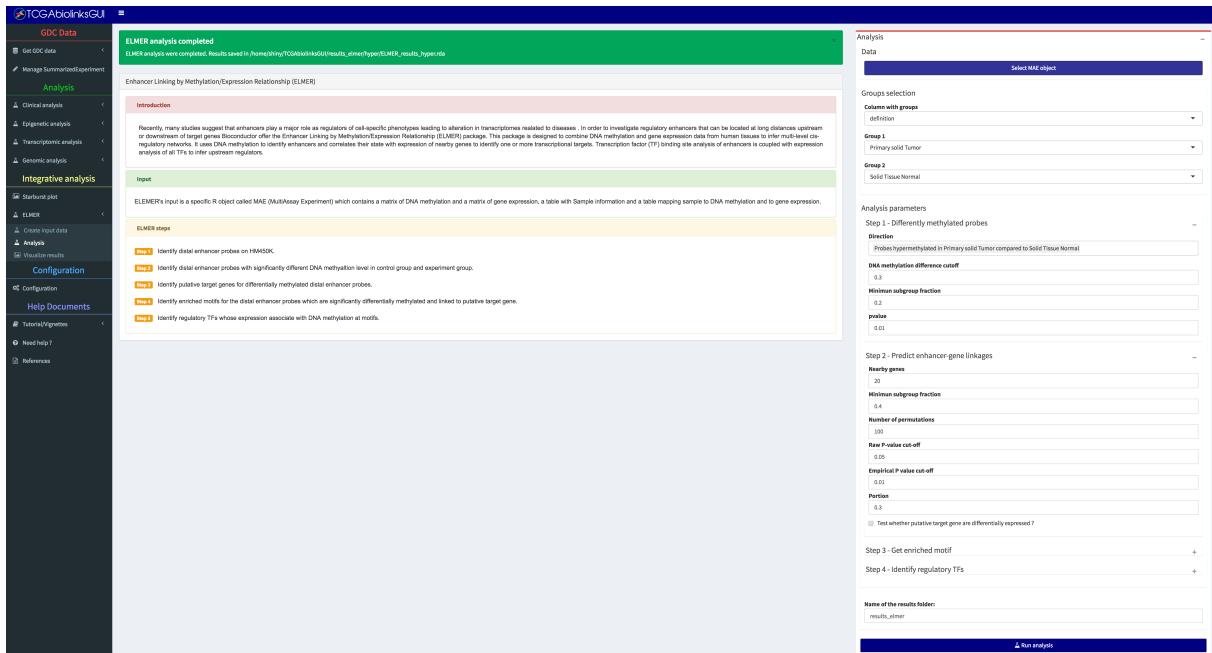


Figure 12 – ELMER analysis menu: Result of ELMER analysis for Primary Solid Tumor vs Solid Tissue Normal of Lung Squamous Cell Carcinoma (LUSC) samples. ELMER searches for probes hypomethylated in Primary Solid Tumor and correlate those regions with the expression of their 20 nearest genes. A motif enrichment analysis is performed on the pairs anti-correlated (loss of DNA methylation and gain of expression in a distal gene), followed by the inference of candidate regulatory transcription factors. If any enriched motifs are found, an object with the results is saved to be later visualized through plots and tables.

### 3.2.4 Docker container

To further simplify the usability and accessibility of our tool, we provide a docker image compatible with most popular operating system available at <https://hub.docker.com/r/tiagochst/tcgabiolinksgui/>. This file allows users to run TCGAbiolinks-GUI without the need to install associated dependencies or configure system files, common steps required to run R installations and load R/Bioconductor packages.

### 3.2.5 Comparison of alternative software

Web tools used for cancer data analysis might be classified into two broad groups. The first group only provides an interface to existing software analysis tools. The Galaxy project (<https://galaxyproject.org/>), which is an open, web-based platform for accessible, reproducible, and transparent computational biomedical research, is an example of such a tool that belongs to this group. The other group is composed of exploratory tools mainly focused on the visualization of processed data and pre-computed results. The cBioPortal project (GAO *et al.*, 2013; CERAMI *et al.*, 2012), by providing several visualizations for mining the TCGA data, is an example of a tool that falls within this classification.

If one were to classify TCGAbiolinksGUI, it would belong to the first group. Compared to the Galaxy project, TCGAbiolinksGUI offers an open platform which improves the accessibility of R/Bioconductor packages, allowing users an advantage to integrate their features with

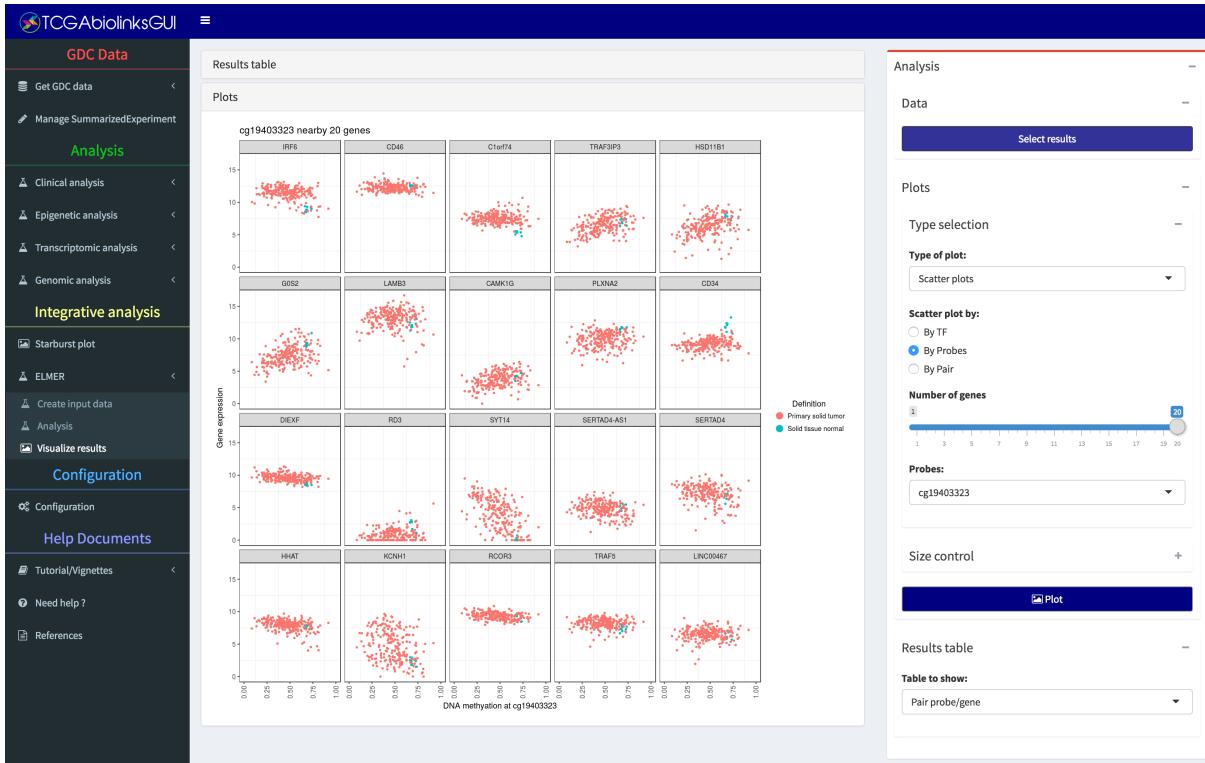


Figure 13 – ELMER visualize results menu: With the result object of ELMER analysis, users are able to visualize scatter plots (expression of 20 nearest genes of a probe vs its DNA methylation level, TF expression vs mean DNA methylation of probes with a given motif, gene expression vs probe DNA methylation level), schematic plots (visualization of pairs of probe and genes), odds ratio (OR) plot (visualization of the motif enrichment analysis results), TF ranking plot (ranking of significance for all TF expression vs mean DNA methylation on the paired probes (binding sites)). Through tables, the user is able to visualize the list of significant differentially methylated probes, significant anti-correlated pairs of genes, enriched motifs and candidate regulatory TFs.

existing Bioconductor packages without the need to go beyond the R/Shiny frameworks as a common feature from the Galaxy project, which requires the interface elements to be structured through XML files (TURAGA *et al.*, 2016). In addition, going beyond the R/Bioconductor environment requires more software dependencies which make the process to install Galaxy to use R/Bioconductor packages laborious. On the other hand, compared to cBioPortal, TCGAbiolinksGUI allows users to perform deep integrative analysis by comparing different subtypes of data (i.e. performing an integrative analysis to compare breast cancer samples with a mutation on FOXA1 gene compared to wild-type samples using DNA methylation, gene expression, and motif enrichment analysis on genomic regions of interest). Although cBioPortal offers these features, it would require users to process each step independently and download outside of cBioPortal in order to perform such integrative analysis.

### 3.3 Enhancer Linking by Methylation/Expression Relationships (ELMER)

Motivated by the discovery of transcriptional enhancers in tissue DNA methylation data (BERMAN *et al.*, 2012), and subsequent approaches to linking these enhancers to transcriptional targets using a chromQTL approach (ARAN; HELLMAN, 2013) (reviewed in Yao *et al.*), Yao *et al.* developed the R/Bioconductor *ELMER* (Enhancer Linking by Methylation/Expression Relationships) package, a tool which infers regulatory element landscapes and transcription factor networks from cancer methylomes.

This tool combined DNA methylation and gene expression data from human tissues to infer multi-level cis-regulatory networks through several steps which included the identification of distal enhancer probes with significantly altered DNA methylation levels in primary tumor tissues compared to normal tissues, followed by the identification of putative target genes, and a comprehensive gene regulatory network analysis which combined transcription factor motifs at the altered enhancers with TF expression to identify the underlying master regulators. This approach identified several known and unknown master regulators in TCGA data, such as GATA-binding protein 3 (GATA3) and Forkhead box protein A1 (FOXA1) in breast cancer, and tumor protein p63 (P63) and Sex determining region Y-box 2 (SOX2) in squamous cell lung carcinoma (YAO *et al.*, 2015; SILVA *et al.*, 2016).

Based on user feedback and a full review of the source code, we identified and implemented a number of software improvements, which are summarized in table 10: (i) The original package contained no standard data structure to handle multiple assays (DNA methylation, gene expression, and clinical data), which would be required for an integrative genomic data analysis. Recently, the Bioconductor team provided such a data structure through the [Multi-AssayExperiment](#) package. (ii) All auxiliary databases (human TF list, classification of TF in families, gene annotation, DNA methylation annotation and motif occurrences within probe sites) used in the package were created and maintained manually, thereby making the upgrade process laborious; thus, we automated this process. (iii) The package was developed to analyze primary tumor tissue samples compared to normal tissues samples, thus not allowing arbitrary subgroups to be compared (for instance mutants vs. non-mutants, treated vs. untreated, etc.) (iv) Our original approach used known epigenomic markers for enhancers to constrain the genomic regions searched for differential methylation. However, this selection could limit our algorithm to identifying regulatory networks for tissue types that exist in the epigenomic databases; we found this constraint problematic, and thus now search *all* distal regulatory regions without any such filter. (v) The function used to download data from The Cancer Genome Atlas (TCGA) data portal (TOMCZAK *et al.*, 2015) broke when the TCGA site was shutdown and its data transferred to The NCI's Genomic Data Commons (GDC) (GROSSMAN *et al.*, 2016); we now have a more general data provider interface that supports GDC as the default provider. (vi)

The package only supported data aligned to Genome Reference Consortium GRCh37 (hg19), and we now provide support for Genome Reference Consortium GRCh38 (hg38). (vii) There was no support to the recent HumanMethylationEPIC (EPIC) array. In addition to the specific improvements listed above, we substantially re-wrote most of the code to be more efficient and maintainable, also most of the output plots generated were improved.

Table 10 – Main differences between ELMER old version (v.1) and the new version (v.2)

| Features                       | ELMER Version 1                                   | ELMER Version 2                                    |
|--------------------------------|---|--|
| Primary data structure         | mee object (custom data structure)                | MAE object (Bioconductor data structure)           |
| Auxiliary data                 | Manually created                                  | Programmatically created                           |
| Number of human TFs            | 1,982   | 1,987 (Uniprot database)                           |
| Number of TF motifs            | 91  | 771 (HOCOMOCO v11 database)                        |
| TF classification              | 78 families                                       | 82 families and 331 subfamilies (TFClass database) |
| Analysis performed             | Normal vs tumor samples                           | Group 1 vs group 2                                 |
| Statistical grouping           | unsupervised only                                 | unsupervised or supervised using labeled groups    |
| TCGA data source               | The Cancer Genome Atlas (TCGA)<br>(not available) | The NCI's Genomic Data Commons (GDC)               |
| Genome of reference            | GRCh37 (hg19)                                     | GRCh37 (hg19)/GRCh38 (hg38)                        |
| DNA methylation platforms      | HumanMethylation450                               | HumanMethylationEPIC and Human-Methylation450      |
| Graphical User interface (GUI) | None  | TCGAbiolinksGUI                                    |

In this section, we present a new version of the R *ELMER* package, which addresses all the issues described above. We start out by the detailed explanation of the algorithms, follow by a case of study using breast Cancer data used as an illustration of the use of the tool.

### 3.3.1 Implementation

Here we describe each of following analysis steps shown in figure 14.

- Organize data as a *MultiAssayExperiment* object
- Identify distal probes with significantly different DNA methylation level when comparing two sample groups.

- Identify putative target genes for differentially methylated distal probes, using methylation vs. expression correlation
- Identify enriched motifs for each probe belonging to a significant probe-gene pair
- Identify master regulatory Transcription Factors (TF) whose expression associate with DNA methylation changes at multiple regulatory regions.

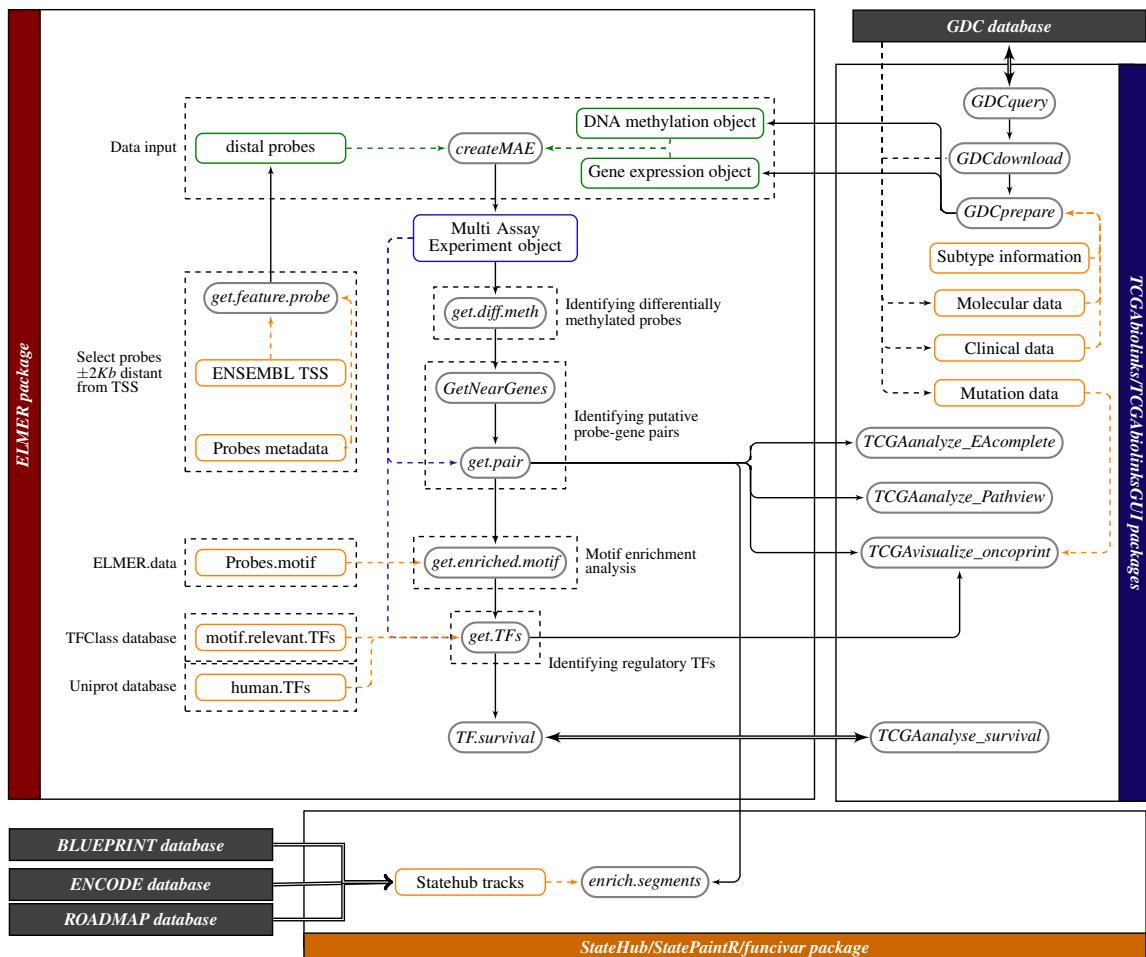


Figure 14 – ELMER workflow: ELMER receives as input a DNA methylation object, a gene expression object (a matrix or a SummarizedExperiment object) and a Genomic Ranges (GRanges) object with distal probes to be used as filter which can be retrieved using the `get.feature.probe` function. The function `createMAE` will create a Multi Assay Experiment object keeping only samples that have both DNA methylation and gene expression data. Genes will be mapped to genomic position and annotated using ENSEMBL database (AKEN *et al.*, 2016), while for probes it will add annotation from ZHOU; LAIRD; SHEN (<http://zwdzwd.github.io/InfiniumAnnotation>). This MAE object will be used as input to the next analysis functions. First, it identifies differentially methylated probes followed by the identification of their nearest genes (10 upstream and 10 downstream) through the `get.diff.meth` and `GetNearGenes` functions respectively. For each probe, it will verify if any of the nearby genes were affected by its change in the DNA methylation level and a list of gene and probes pairs will be outputted from `get.pair` function. For the probes in those pairs, it will search for enriched regulatory Transcription Factors motifs with the `get.enriched.motif` function. Finally, the enriched motifs will be correlated with the level of the transcription factor through the `get.TFs` function. In the figure green Boxes represents user input data, blue boxes represents output object, orange boxes represent auxiliary pre-computed data and gray boxes are functions.

### 3.3.1.1 Organization of data as a *MultiAssayExperiment* object

To facilitate the analysis of experiments and studies with multiple samples the Bioconductor team created the *SummarizedExperiment* class (HUBER *et al.*, 2015), a data structure able to store data and metadata for a single experiment but not for data spanning several experiments for the same sample. To overcome this problem, recently, the MultiAssay SIG (Special Interest Group) created the *MultiAssayExperiment class* (SIG, 2017) a data structure to manage and preprocess multiple assays for integrated genomic analysis. This data structure is now an input for all main functions of *ELMER* and can be generated by the *createMAE* function.

To perform *ELMER* analyses, users need to populate a *MultiAssayExperiment* with a DNA methylation matrix or *SummarizedExperiment* object from HumanMethylation450 BeadChip (HM450) or MethylationEPIC BeadChip (EPIC) platform; a gene expression matrix or *SummarizedExperiment* object for the same samples; a matrix mapping DNA methylation samples to gene expression samples; and a matrix with sample metadata (i.e. clinical data, molecular subtype, etc.). If TCGA data are used, the last two matrices will be automatically generated. If using non-TCGA data, the matrix with sample metadata should be provided with at least a column with a patient identifier and another one identifying its group which will be used for analysis, if samples in the methylation and expression matrices are not ordered and with same names, a matrix mapping for each patient identifier their DNA methylation samples and their gene expression samples should be provided to the *createMAE* function. Based on the genome of reference selected, metadata for the DNA methylation probes, such as genomic coordinates, will be added from Zhou, Laird e Shen (2016); and metadata for gene expression and annotation is added from ENSEMBL database (YATES *et al.*, 2015) using *biomaRt* (DURINCK *et al.*, 2009).

### 3.3.1.2 Selecting distal probes

Probes from HumanMethylationEPIC (EPIC) array and Infinium HumanMethylation450 (HM450) array are removed from the analysis if they have either internal SNPs close to the 3' end of the probe; non-unique mapping to the bisulfite-converted genome; or off-target hybridization due to partial overlap with non-unique elements (ZHOU; LAIRD; SHEN, 2017). This probe metadata information is included in *ELMER.data* package, populated from the source file at <http://zwdzwd.github.io/InfiniumAnnotation> (ZHOU; LAIRD; SHEN, 2017). To limit ELMER to the analysis of distal elements, probes located in regions of  $\pm 2kb$  around transcription start sites (TSSs) were removed.

### 3.3.1.3 Identification of differentially methylated CpGs (DMCs)

For each distal probe, samples of each group (group 1 and group 2) are ranked by their DNA methylation beta values, those samples in the lower quintile (20% samples with the lowest methylation levels) of each group are used to identify if the probe is hypomethylated in group 1 compared to group 2, using an unpaired one-tailed t-test. The 20% is a parameter to the *diff.meth*

function called *minSubgroupFrac*. For the (ungrouped) cancer case, this is set to 20% as in Yao *et al.* (2015), because we typically wanted to be able to detect a specific molecular subtype among the tumor samples; these subtypes often make up only a minority of samples, and 20% was chosen as a lower bound for the purposes of statistical power (high enough sample numbers to yield t-test p-values that could overcome multiple hypothesis corrections, yet low enough to be able to capture changes in individual molecular subtypes occurring in 20% or more of the cases.) This number can be set arbitrarily as an input to the *diff.meth* function and should be tuned based on sample sizes in individual studies. In the *Supervised* mode, where the comparison groups are implicit in the sample set and labeled, the *minSubgroupFrac* parameter is set to 100%. An example would be a cell culture experiment with 5 replicates of the untreated cell line, and another 5 replicates that include an experimental treatment.

To identify hypomethylated differentially methylated CpGs (DMCs), a one-tailed t-test is used to rule out the null hypothesis:  $\mu_{group1} \geq \mu_{group2}$ , where  $\mu_{group1}$  is the mean methylation within the lowest group 1 quintile (or another percentile as specified by the *minSubgroupFrac* parameter) and  $\mu_{group2}$  is the mean within the lowest group 2 quintile. Raw p-values are adjusted for multiple hypothesis testing using the Benjamini-Hochberg method (BENJAMINI; HOCHBERG, 1995b), and probes are selected when they had adjusted p-value less than 0.01 (which can be configured using the *pvalue* parameter). For additional stringency, probes are only selected if the methylation difference:  $\Delta = \mu_{group1} - \mu_{group2}$  was greater than 0.3. The same method is used to identify hypermethylated DMCs, except we use the *upper* quintile, and the opposite tail in the t-test is chosen.

#### *Identification of putative target gene(s)*

For each differentially methylated distal probe (DMC), the closest 10 upstream genes and the closest 10 downstream genes are tested for inverse correlation between methylation of the probe and expression of the gene (the number 10 can be changed using the *numFlankingGenes* parameter). To select these genes, the probe-gene distance is defined as the distance from the probe to the transcription start site specified by the ENSEMBL gene level annotations (YATES *et al.*, 2015) accessed via the R/Bioconductor package [biomaRt](#) (DURINCK *et al.*, 2009; DURINCK *et al.*, 2005). By choosing a constant number of genes to test for each probe, our goal is to avoid systematic false positives for probes in gene rich regions. This is especially important given the highly non-uniform gene density of mammalian genomes. Thus, exactly 20 statistical tests were performed for each probe, as follows.

For each probe-gene pair, the samples (all samples from both groups) are divided into two groups: the *M* group, which consisted of the upper methylation quintile (the 20% of samples with the highest methylation at the enhancer probe), and the *U* group, which consists of the lowest methylation quintile (the 20% of samples with the lowest methylation.) The 20% ile cutoff is a configurable parameter *minSubgroupFrac* in the *get.pair* function. As with its usage

in the *diff.meth* function, the default value of 20% is a balance, allowing for the identification of changes in a molecular subtype making up a minority (i.e. 20%) of cases, while also yielding enough statistical power to make strong predictions. For larger sample sizes or other experimental designs, this could be set even lower.

For each candidate probe-gene pair, the Mann-Whitney U test is used to test the null hypothesis that overall gene expression in group M is greater than or equal than that in group U. This non-parametric test was used in order to minimize the effects of expression outliers, which can occur across a very wide dynamic range. For each probe-gene pair tested, the raw p-value  $P_r$  is corrected for multiple hypothesis using a permutation approach as follows. The gene in the pair is held constant, and  $x$  random methylation probes are chosen to perform the same one-tailed U test, generating a set of  $x$  permutation p-values  $P_p$ . We chose the  $x$  random probes only from among those that were "distal" (farther than 2kb from an annotated transcription start site), in order to draw these null-model probes from the same set as the probe being tested (SHAM; PURCELL, 2014). An empirical p-value  $P_e$  value was calculated using the following formula (which introduces a pseudo-count of 1):

$$P_e = \frac{\text{num}(P_p \leq P_r) + 1}{x + 1} \quad (3.1)$$

Notice that in the *Supervised* mode, no additional filtering is necessary to ensure that the M and U group segregate by sample group labels. The two sample groups are segregated by definition, since these probes were selected for their differential methylation, with the same directionality, between the two groups.

### 3.3.1.4 Characterization of chromatin state context of enriched probes using FunciVar

Unlike version 1 of *ELMER*, we now consider *all* distal probes in the identification of regulatory elements. DNA methylation is known to affect several different classes of distal chromatin state element, including active enhancers, poised enhancers, and insulators. In order to provide a functional interpretation of the regulatory elements identified by *ELMER*, we perform a chromatin state enrichment analysis of the probes within significant probe-gene pairs, using the *statePaintR* tools from the [www.statehub.org](http://www.statehub.org) (COETZEE *et al.*, 2017), along with our new FunciVar package (FUNCIRVAR, ). Enrichment of the putative pairs within chromatin states is calculated against a background model that uses the distal probe set that the putative pairs are drawn from.

#### Motif enrichment analysis

In order to identify enriched motifs and potential upstream regulatory TFs, all probes with occurring in significant probe-gene pairs are combined for motif enrichment analysis. Hypergeometric Optimization of Motif EnRichment (HOMER) (HEINZ *et al.*, 2010) is used

to find motif occurrences in a  $\pm 250\text{bp}$  region around each probe, using HOCOMOCO (HOmo sapiens COmprehensive MOdel Collection) v11 (KULAKOVSKIY *et al.*, 2016). Transcription factor (TF) binding models are available at <http://hocomoco.autosome.ru/downloads> (using the HOMER specific format with threshold score levels corresponding to p-value  $\leq 1^{-4}$ ).

For each probe set tested (i.e. the set of all probes occurring in significant probe-gene pairs), a motif enrichment Odds Ratio and a 95% confidence interval are calculated using following formulas:

$$p = \frac{a}{a+b} \quad (3.2a)$$

$$P = \frac{c}{c+d} \quad (3.2b)$$

$$\text{OddsRatio} = \frac{\frac{p}{(1-p)}}{\frac{P}{1-P}} = \frac{p(1-P)}{P(1-p)} = \frac{ad}{bc} \quad (3.2c)$$

$$SD = \sqrt{\frac{1}{a} + \frac{1}{b} + \frac{1}{c} + \frac{1}{d}} \quad (3.2d)$$

where  $a$  is the number of probes within the selected probe set that contains one or more motif occurrences;  $b$  is the number of probes within the selected probe set that do not contain a motif occurrence;  $c$  and  $d$  are the same counts within the entire array probe set (drawn from the same set of distal-only probes using the same definition as the primary analysis). A probe set was considered significantly enriched for a particular motif if the 95% confidence interval of the Odds Ratio was greater than 1.1 (specified by option *lower.OR*, 1.1 is default), and the motif occurred at least 10 times (specified by option *min.incidence*, 10 is default) in the probe set.

### 3.3.1.5 Identification of master regulator TFs

When a group of enhancers is coordinately altered in a specific sample subset, this is often the result of an altered upstream *master regulator* transcription factor in the gene regulatory network. *ELMER* tries to identify such transcription factors corresponding to each of the TF binding motifs enriched from the previous analysis step. For each enriched motif, *ELMER* takes the average DNA methylation of all distal probes (in significant probe-gene pairs) that contain that motif occurrence (within a  $\pm 250\text{bp}$  region) and compares this average DNA methylation to the expression of each gene annotated as a human TF.

A statistical test is performed for each motif-TF pair, as follows. All samples are divided into two groups: the  $M$  group, which consists of the 20% of samples with the highest average methylation at all motif-adjacent probes, and the  $U$  group, which consisted of the 20% of samples with the lowest methylation. This step is performed by the *get.TFs* function, which takes *minSubgroupFrac* as an input parameter, again with a default of 20%. For each candidate motif-TF pair, the Mann-Whitney U test is used to test the null hypothesis that overall gene expression in group  $M$  is greater or equal than that in group  $U$ . This non-parametric test was used in order to minimize the effects of expression outliers, which can occur across a very wide

dynamic range. For each motif tested, this results in a raw p-value ( $P_r$ ) for each of the human TFs. All TFs are ranked by their  $-\log_{10}(Pr)$  values, and those falling within the top 5% of this ranking were considered candidate upstream regulators. The best upstream TFs which are known to recognize to specific binding motif are automatically extracted as putative regulatory TFs, and rank ordered plots are created to visually inspect these relationships, as shown in the example below. Because the same motif can be recognized by many transcription factors of the same binding domain family, we define these relationships at both the family and subfamily classification level using the classifications from TFClass database (WINGENDER; SCHOEPS; DÖNITZ, 2013). Use of this database is a major change from version 1 of ELMER, which used custom curations for DNA binding domain families. Use of the TFClass database is preferable because it is well curated and regularly updated to reflect new findings.

### 3.3.2 Use Case 1: Breast Invasive Carcinoma (unsupervised approach)

In this subsection, we describe how to perform *ELMER* analysis on TCGA BRCA (Breast Invasive Carcinoma) data retrieved from the GDC server. We first describe how the data can be downloaded and organized to the default *ELMER* input, followed by the following analysis steps:

- Identification of distal probes with significant differential DNA methylation (i.e. DMCs) in tumor vs. normal samples
- Identification of putative target gene(s) for differentially methylated distal probes
- Characterization of chromatin state context of significant probe regions using FunciVar
- Identification of enriched motifs within set of probes in significant probe-gene pairs
- Identification of master regulator Transcription Factors (TF) for each enriched motif

In addition to these standard steps, we also compared the putative probe-gene pairs to those derived from deep-sequenced ChIA-PET data from MCF7 cells (as shown in (YAO *et al.*, 2015)).

#### *Downloading TCGA data*

The function `getTCGA` uses the [TCGAbiolinks](#) package (COLAPRICO *et al.*, 2015) to download TCGA data for all samples for a given disease (such as BLCA, LGG, GBM). Its main arguments are the *genome* that if set to "hg19" will download data from GDC legacy archive, and if set to "hg38" it will download data from the main GDC harmonized data portal.

If the `getTCGA` function called before was successful it will create the following objects and folders:

```

--- DATA/BRCA/
|----- BRCA_meth_hg38.rda (object with DNA methylation)
|----- BRCA_RNA_hg38.rda (object with gene expression)
|----- BRCA_clinic.rda (object with indexed clinical information)
|----- Raw/ (folder: contains All raw data from GDC)

```

### Selecting distal probes

The function `get.feature.probe`, shown in Listing 1, is used to select HM450K/EPIC probes located away from any TSS (at least 2Kb away). Its main arguments are the genome of reference ("hg38"/"hg19") and DNA methylation platform ("450K"/"EPIC"). The `feature` argument is used to limit the region of probes; as we want all distal probes, we set it to NULL.

#### Source code 1 – "Selection of probes within biofeatures"

```

1 # get distal probes that are 2kb away from TSS
2 distal.probes <- get.feature.probe(feature = NULL,
3                                     genome = "hg38",
4                                     met.platform = "450K")
5 # 168644 probes

```

### Organizing data into a MultiAssayExperiment object

The function `createMAE` is used to organize the gene expression and DNA methylation data into a MultiAssayExperiment (MAE) object. Listing 2 shows how to use it with the data created in the previous steps. Its main arguments are described below:

- `exp` : An R object or a path to a file containing a gene expression matrix or SummarizedExperiment with gene counts.
- `met` : An R object or a path to a file containing a DNA methylation matrix or SummarizedExperiment with beta values.
- `met.platform`: DNA methylation platform. "EPIC" for Infinium MethylationEPIC or "450K" for Infinium HumanMethylation450.
- `genome`: The genome of reference ("hg19" or "hg38") used to select the correct metadata. Genes genomic ranges will be annotated using ENSEMBL database and DNA methylation probes using metadata available at <http://zwdzwd.github.io/InfiniumAnnotation>.
- `linearize.exp`: this step will take the  $\log_2(gene\ expression + 1)$  in order to linearize the relationship between gene expression and DNA methylation.
- `filter.probes`: genomic ranges (i.e. distal regions) within which probes from DNA methylation data should be kept.

- *met.na.cut*: maximum percentage of empty values (NA) a probe might have to be considered in the analysis. The default is 20% (i.e if 50% of samples has empty values for a given probe, it will be removed).
- *colData*: A matrix with samples metadata (i.e. clinical data , molecular subtype information). If argument TCGA is set to *TRUE* this matrix will be created automatically. In this case, the *colData* argument is optional.
- *sampleMap*: A matrix mapping DNA methylation data and gene expression data to samples. *ELMER* uses only samples with both data. Otherwise, it will be removed. If argument TCGA is set to *TRUE* this matrix will be created automatically. In this case *sampleMap* argument is optional.

#### Source code 2 – "Create MultiAssayExperiment"

```

1
2 mae <- createMAE(exp = "DATA/BRCA/BRCA_RNA_hg38.rda",
3                     met = "DATA/BRCA/BRCA_meth_hg38.rda",
4                     met.platform = "450K",
5                     genome = "hg38",
6                     linearize.exp = TRUE,
7                     filter.probes = distal.probes,
8                     met.na.cut = 0.2,
9                     save = TRUE,
10                    TCGA = TRUE)

```

Listing 3 shows information about the object created. There are 866 samples with both gene expression and DNA methylation data, and among those 5 are metastatic samples, 778 are Primary Solid Tumor and 83 are Solid Tissue Normal.

#### Source code 3 – "Verifying MultiAssayExperiment"

```

1
2 > mae
3 A MultiAssayExperiment object of 2 listed
4 experiments with user-defined names and respective classes.
5 Containing an ExperimentList class object of length 2:
6 [1] DNA methylation: RangedSummarizedExperiment with 135331 rows and
    866 columns
7 [2] Gene expression: RangedSummarizedExperiment with 57035 rows and
    866 columns
8 Features:
9 experiments() - obtain the ExperimentList instance
10 colData() - the primary/phenotype DataFrame
11 sampleMap() - the sample availability DataFrame
12 '$', '[', '[' - extract colData columns, subset, or experiment
13 *Format() - convert ExperimentList into a long or wide DataFrame

```

```

14 assays() - convert ExperimentList to a list of rectangular matrices
15 > table(mae$definition)
16      Metastatic Primary solid Tumor Solid Tissue Normal
17          5             778            83

```

### ***Identification of distal probes with significant differential DNA methylation (i.e. DMCs) in tumor vs. normal samples***

The function `get.diff.meth` is used to identify regions differently methylation between two groups. Listing 4 shows how to use it to select hypomethylated probes in "Primary solid tumor" samples when compared to "solid tissue normal" samples ( $FDR \leq 0.01$ ,  $\Delta\bar{\beta} \geq 0.3$ ), using those samples in the lower quintile ( $minSubgroupFrac = 0.2$ ) of DNA methylation levels for each probe. Its main arguments are described below:

- *data* A multiAssayExperiment with DNA methylation and Gene Expression data.
- *group.col* A column defining the groups of the sample. You can view the available columns using: `colnames(MultiAssayExperiment::colData(data))`.
- *group1* A group from *group.col*. *ELMER* will run *group1* vs *group2*. That means, if the direction is hyper, get probes hypermethylated in group 1 compared to group 2.
- *group2* A group from *group.col*. *ELMER* will run *group1* vs *group2*. That means, if the direction is hyper, get probes hypermethylated in group 1 compared to group 2.
- *diff.dir* Differential methylation direction. It can be "hypo" which is only selecting hypomethylated probes in group 1 when compared to group 2; "hyper" which is only selecting hypermethylated probes;
- *minSubgroupFrac* A number ranging from 0 to 1, specifying the fraction of extreme samples from group 1 and group 2 that are used to identify the differential DNA methylation. The default is 0.2 because we typically want to be able to detect a specific (possibly unknown) molecular subtype among tumor; these subtypes often make up only a minority of samples, and 20% was chosen as a lower bound for the purposes of statistical power. If you are using pre-defined group labels, such as treated replicates vs. untreated replicated, use a value of 1.0 (*Supervised* mode)
- *pvalue* A number specifying the significant P value (adjusted P value by Benjamini-Hochberg procedure) cutoff for selecting significant hypo/hyper-methylated probes. The default is 0.01.
- *sig.dif* A number specifying the smallest DNA methylation difference as a cutoff for selecting significant hypo/hyper-methylated probes. The default is 0.3.

Source code 4 – "Identify significantly different DNA methylation probes in tumor and normal samples"

```

1 diff.probes <- get.diff.meth(data = mae,
2                               group.col = "definition",
3                               group1 = "Primary solid Tumor",
4                               group2 = "Solid Tissue Normal",
5                               diff.dir = "hypo", # Get probes hypometh.
6                               in.group = 1
7                               cores = 1,
8                               minSubgroupFrac = 0.2, # % group samples
9                               used.
10                              pvalue = 0.01,
11                              sig.dif = 0.3,
12                              dir.out = "Results_hypo/",
13                              save = TRUE)

```

If the *save* argument is set to TRUE, in the *dir.out* folder two files will be created: *getMethdiff.hypo.probes.csv* containing all probes from the DNA methylation data with the difference means of the groups and the significance values, *getMethdiff.hypo.probes.significant.csv* will contain only probes that respect the thresholds. Table 11 shows the first rows of *getMethdiff.hypo.probes.significant.csv* file.

| probe      | pvalue   | Primary.solid.Tumor_Minus_Solid.Tissue.Normal | adjust.p |
|------------|----------|---|----------|
| cg00001809 | 1.97e-35 | -0.32   | 1.26e-34 |
| cg00008695 | 1.62e-67 | -0.44   | 3.72e-66 |
| cg00009553 | 6.84e-31 | -0.525  | 3.61e-30 |

Table 11 – Identification of distal probes with significant differential DNA methylation (i.e. DMCs): First three rows of *getMethdiff.hypo.probes.significant.csv* file.

## **Identification of putative target gene(s) for differentially methylated distal probes**

The function `get.pair` is used to link enhancer probes with methylation changes to target genes with expression changes and report the putative target gene for selected probes. Listing 5 shows how to select the 20 nearest genes (10 downstream and 10 upstream) and evaluate if each pair is anti-correlated (probes with higher methylation levels have lower gene expression levels). Its main arguments are described below:

- `nearGenes` Output of `GetNearGenes` function.
- `mode` Algorithm mode: "unsupervised" or "supervised". If unsupervised is set the U (unmethylated) and M (methylated) groups will be selected among all samples of both groups based on methylation of each probe. Otherwise U group and M group will set as all the samples of group1 or group2 as described below: If `diff.dir` is "hypo", U will be the group 1 and M the group2. If `diff.dir` is "hyper" M group will be the group1 and U the group2.
- `minSubgroupFrac` A number ranging from 0 to 1, specifying the fraction of extreme samples that define group U (unmethylated) and group M (methylated), which are used to link probes to genes. The default is 0.4 (the lowest quintile of samples is the U group and the highest quintile samples is the M group) because we typically want to be able to detect a specific (possibly unknown) molecular subtype among tumor; these subtypes often make up only a minority of samples, and 20% was chosen as a lower bound for the purposes of statistical power. This argument is Only used if mode is "supervised", otherwise if you are using pre-defined group labels ("supervised" mode), such as treated replicates vs. untreated replicated, it will use all samples.
- `permu.size` Number of permutation. The default is 10000. *Note:* This parameter can strongly impact run time.
- `raw.pvalue` Raw p-value cutoff for defining significant pairs. The default is 0.001.
- `Pe` Empirical p-value cutoff for defining significant pairs. The default is 0.001.
- `filter.probes` Should probes be filtered by selecting only those which have at least a certain number of samples below and above a certain cut-off? If true, arguments `filter.probes` and `filter.percentage` will be used.
- `filter.portion` A number specifying the cut point to define binary methylation level for probe loci. The default is 0.3. When the beta value is above 0.3, the probe is methylated and vice versa. For one probe, the percentage of methylated and unmethylated samples should be above `filter.percentage` value. Only used if `filter.probes` is TRUE.

- *filter.percentage* Minimum percentage of samples to be considered in methylated and unmethylated for the filter.portion option. Default 5%. Only used if *filter.probes* is TRUE.

Source code 5 – "Identify putative target genes for differentially methylated distal probes"

```

1
2 # For each differently methylated probes we will get the
3 # 20 nearby genes (10 downstream and 10 upstream)
4 nearGenes <- GetNearGenes(data = mae,
5                               probes = diff.probes$probe,
6                               numFlankingGenes = 20,
7                               cores = 1)
8
9 # This step is the most time consuming. Depending on the size of the
# groups
10 # and the number of probes found previously it might take hours
11 Hypo.pair <- get.pair(data = mae,
12                        nearGenes = nearGenes,
13                        group.col = "definition",
14                        group1 = "Primary solid Tumor",
15                        group2 = "Solid Tissue Normal",
16                        permu.dir = "Results_hypo/permu",
17                        permu.size = 10000,
18                        mode = "unsupervised",
19                        minSubgroupFrac = 0.4, # 40% of samples to
# create U and M
20                        raw.pvalue = 0.001,
21                        Pe = 0.001,
22                        filter.probes = TRUE,
23                        filter.percentage = 0.05,
24                        filter.portion = 0.3,
25                        dir.out = "Results_hypo",
26                        cores = 1,
27                        label = "hypo")
28 # Number of pairs: 2950

```

The output of this function is shown in table 12. Probe and GeneID columns show the significant pair and the column  $P_e$  shows the adjusted p-value.

| Probe      | GeneID          | Symbol  | Distance | Sides | Raw.p    | Pe        |
|------------|-----------------|---------|----------|-------|----------|-----------|
| cg14058239 | ENSG00000141424 | SLC39A6 | 0        | R1    | 5.17e-56 | 9.99e-5   |
| cg14986386 | ENSG00000183323 | CCDC125 | 0        | L2    | 3.26e-55 | 9.99e-5   |
| cg04723436 | ENSG00000107485 | GATA3   | 110455   | R3    | 6.00e-55 | 4.9.99e-4 |

Table 12 – Identification of putative target gene(s) for differentially methylated distal probes: First three rows of getPair.hypo.pairs.significant.csv file.

To visualize the relationship between the probe-gene pairs inferred, there are two auxiliary functions in ELMER. The function *schematic.plot*, shown in Listing 6, which will plot genes and probes in a specified genomic region, highlighting the significant pairs identified by plotting a genomic interactions track and highlight the genes in the pair in red (Figure 15). Also, using the function *scatter.plot* (Listing 7) it is possible to visualize the correlation between gene expression and DNA methylation levels at probe (Figure 16).

Source code 6 – "Schematic plot to visualize gene-probe pairs"

```
1  
2 # by probe and with detail about DNA methylation  
3 schematic.plot(data = mae,  
4                  group.col = "definition",  
5                  group1 = "Primary solid Tumor",  
6                  group2 = "Solid Tissue Normal",  
7                  pair = Hypo.pair,  
8                  statehub.tracks = "hg38/ENCODE/mcf-7.16mark.  
9                  segmentation.bed",  
9                  byProbe = "cg04723436")
```

Source code 7 – "Scatter plot to visualize correlation between gene expression and DNA methylation levels at probe"

```
1  
2 scatter.plot(data = mae,  
3                 byPair = list(probe = "cg04723436",  
4                               gene = "ENSG00000107485"),  
5                 save = T,  
6                 category = "definition",  
7                 lm = TRUE)
```

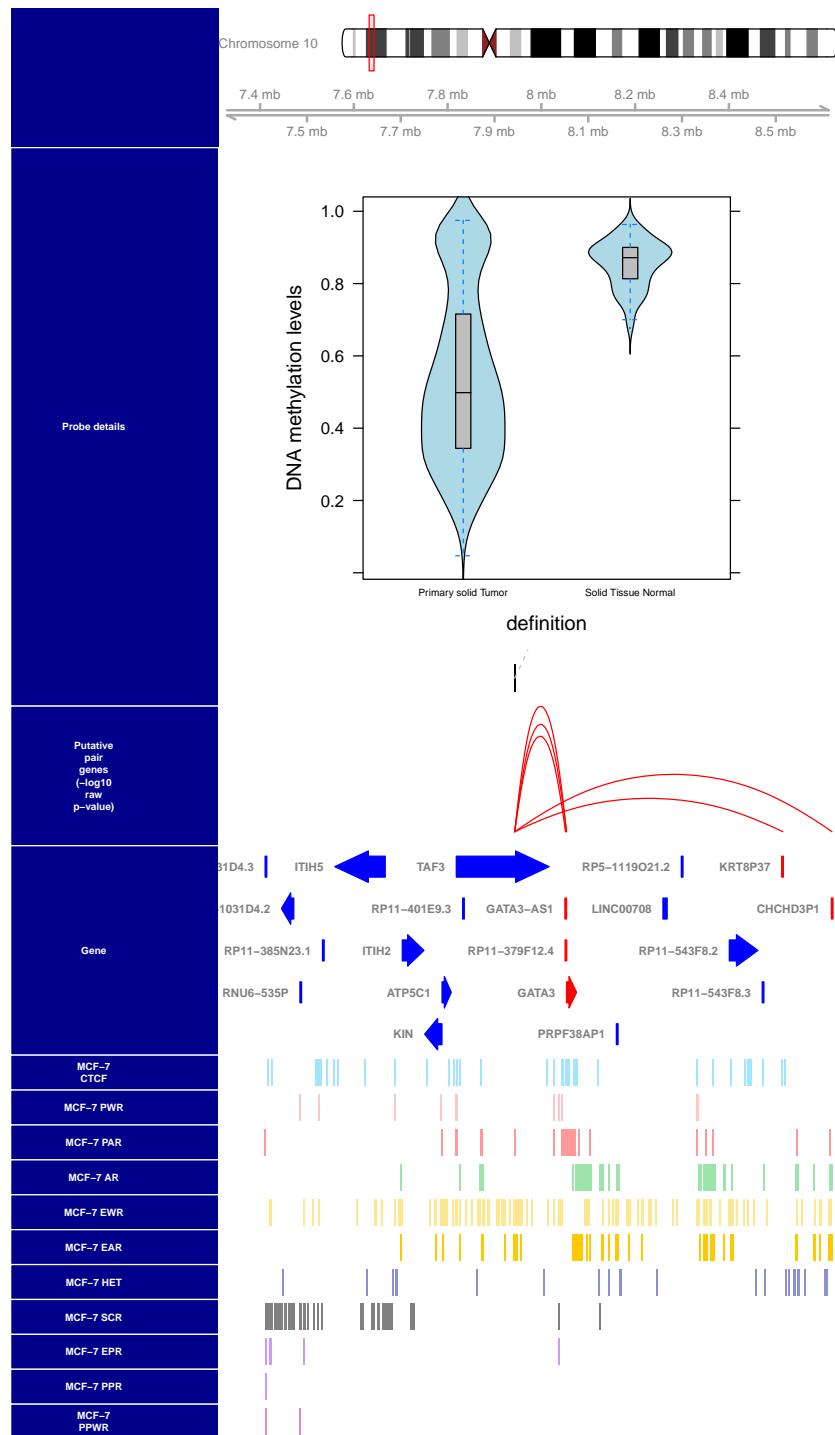


Figure 15 – Plot probe-gene pairs with annotation track for MCF-7 cell line from [StateHub.org](http://StateHub.org). Significant probes and gene pairs are highlighted in red.

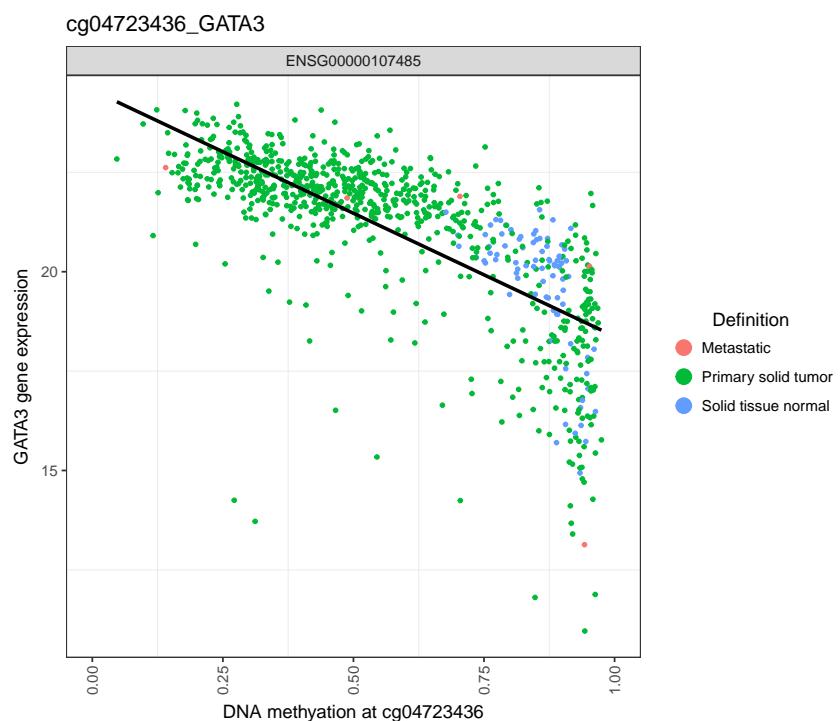


Figure 16 – Scatter plot for significant probe (cg04723436) gene (GATA3) pair.

## Characterization of chromatin state context of significant probe regions using FunciVar

To understand and compare our set of probes identified in the probe-gene pairs inferred we used chromatin state of IHEC cell types from <http://statehub.org/>, to calculate the relative enrichment of different states. This procedure uses code from the statepaintR (COETZEE *et al.*, 2017) and FunciVar (FUNCIRVAR, ) packages. Figure 17 shows the enrichment for 14 encode cells lines. The plot shows enrichment for enhancer active region (EAR), weak enhancer (EWR) and active promoter region (PAR) in MCF-7 cell (human breast adenocarcinoma cell line) while for other cell lines this enrichment is not visible.

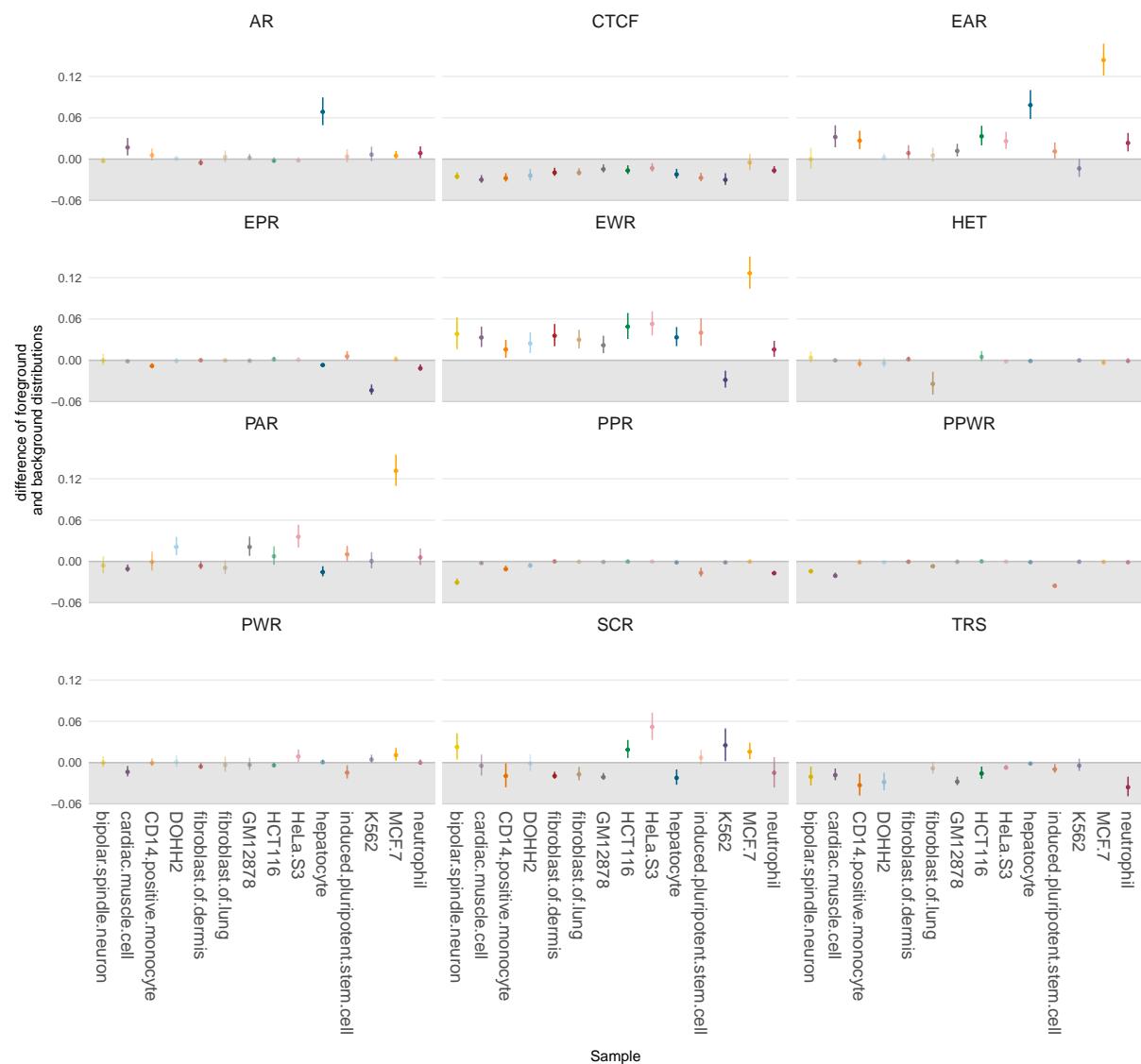


Figure 17 – Enrichment of paired probes and chromatin states of encode cells. The plot shows enrichment for enhancer active region, weak enhancer and active promoter region for MCF-7 cell. Acronyms - AR: Active region, EAR: active enhancer, EWR: Weak Enhancer, EPR: poised enhancer, PAR: active promoter, PWR: Weak Promoter, PPR: poised promoter, PPWR: Weak Poised Promoter, CTCF: architectural complex, TRS: transcribed, HET: heterochromatin, SCR: Polycomb Repressed Silenced

## ***Identification of enriched motifs within set of probes in significant probe-gene pairs***

The function `get.enriched.motif` is used to identify enriched motif in a set of probes. The main arguments are described below:

- *lower.OR* The motif with lower boundary of 95% confidence interval for Odds Ratio  $\geq lower.OR$  are the significantly enriched motifs.
  - *min.incidence* Minimum number of probes having the motif signature (default: 10) required for a motif to be enriched.

## Source code 8 – "Motif enrichment analysis on the selected probes"

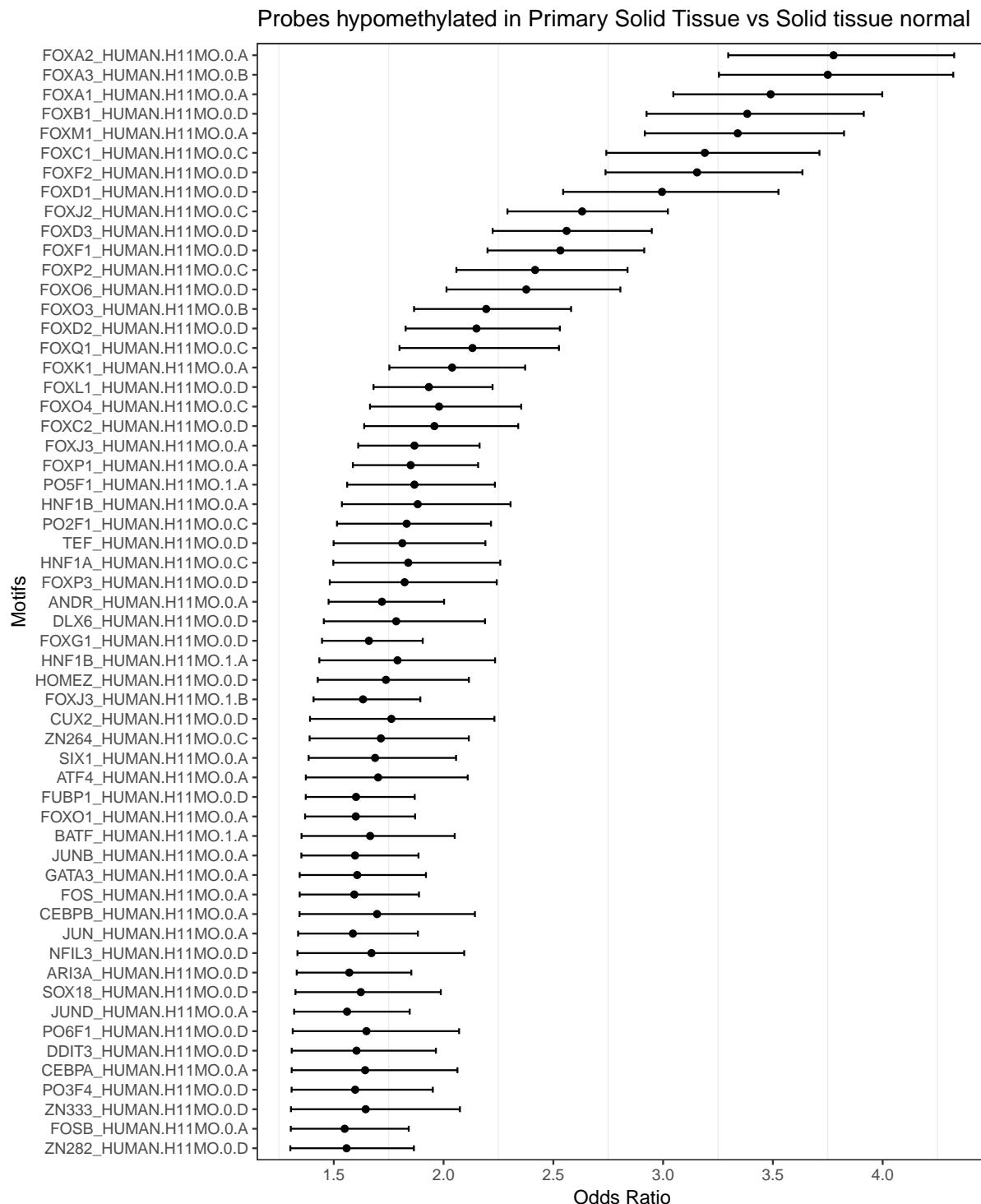


Figure 18 – Motif enrichment plot shows the enrichment levels ( $OR \geq 2.0$ ) for the selected motifs. This plot represents a subset of the enriched motifs for  $lower.or = 1.1$ , only selected for representational purposes.

### **Identification of master regulator Transcription Factors (TF) for each enriched motif**

The function `get.TFs` is used to identify regulatory TF whose expression associates with TF binding motif DNA methylation which.

Source code 9 – "Identifying regulatory Transcript Factors"

```

1
2 TF <- get.TFs(data = mae,
3                   group.col = "definition",
4                   group1 = "Primary solid Tumor",
5                   group2 = "Solid Tissue Normal",
6                   minSubgroupFrac = 0.4, # Set to 1 if supervised mode
7                   enriched.motif = enriched.motif,
8                   dir.out = "Results_hypo",
9                   cores = 1,
10                  label = "hypo")
11
12 paste(sort(unique(TF$top.potential.TF.family))),collapse = ",")#
13 # "EMX1 ,ESR1 ,FOXA1 ,GATA3 ,HOMEZ ,LMX1B ,MYB ,MZF1 ,NR2F6 ,OVOL1 ,PBX1 ,RARA ,
14 SPDEF ,ZKSCAN1 ,ZSCAN16"
14 paste(sort(unique(TF$top.potential.TF.subfamily))),collapse = ",")#
15 # "AR ,EMX1 ,FOXA1 ,FOXD2 ,GATA3 ,HOMEZ ,LMX1B ,MYB ,NR2E3 ,PBX1 ,ZKSCAN1 ,
15 ZSCAN16"

```

The result of this function is shown in table 13 and in figure 19.

| motif   | top.potential.TF.family | top.potential.TF.subfamily | potential.TF.family | potential.TF.subfamily | top_5percent_TFs                 |
|---------|-------------------------|----------------------------|---------------------|------------------------|----------------------------------|
| AIRE.C  | NA                      | NA                         | NA                  | NA                     | FOXA1;GATA3;ESR1;SPDEF;RARA;...  |
| ANDR.A  | ESR1                    | AR                         | ESR1;AR             | AR                     | FOXA1;GATA3;ESR1;RARA;SPDEF;...  |
| ANDR2.A | ESR1                    | AR                         | ESR1;AR             | AR                     | FOXA1;GATA3;ESR1;RARA;SPDEF;...  |
| BSH.D   | EMX1                    | NA                         | EMX1;LBX2           | NA                     | FOXA1;GATA3;ESR1;SPDEF;RARA;...  |
| CDC5L.D | MYB                     | MYB                        | MYB                 | MYB                    | FOXA1;GATA3;ESR1;SPDEF;RARA;...  |
| DLX2.D  | EMX1                    | NA                         | EMX1;LBX2           | NA                     | FOXA1;GATA3;ESR1;SPDEF;CXXC5;... |
| EMX1.D  | EMX1                    | EMX1                       | EMX1;LBX2           | EMX1                   | FOXA1;GATA3;ESR1;SPDEF;CXXC5;... |
| EMX2.D  | EMX1                    | EMX1                       | EMX1;LBX2           | EMX1                   | FOXA1;GATA3;ESR1;SPDEF;CXXC5;... |
| ERR3.B  | ESR1                    | ESR1                       | ESR1;AR             | ESR1                   | FOXA1;GATA3;ESR1;SPDEF;CXXC5;... |
| EVI1.B  | ZNF467                  | NA                         | ZNF467;PATZ1        | NA                     | FOXA1;GATA3;ESR1;SPDEF;RARA;...  |
| EVX1.D  | NA                      | NA                         | NA                  | NA                     | FOXA1;GATA3;ESR1;CXXC5;SPDEF;... |
| FOSB.A  | NA                      | NA                         | NA                  | NA                     | FOXA1;GATA3;ESR1;RARA;CXXC5;...  |
| FOSL1.A | NA                      | NA                         | NA                  | NA                     | FOXA1;GATA3;ESR1;CXXC5;RARA;...  |
| FOSL2.A | NA                      | NA                         | NA                  | NA                     | FOXA1;GATA3;ESR1;CXXC5;RARA;...  |
| FOS.A   | NA                      | NA                         | NA                  | NA                     | FOXA1;GATA3;ESR1;CXXC5;RARA;...  |
| FOXA1.A | FOXA1                   | FOXA1                      | FOXA1               | FOXA1                  | FOXA1;GATA3;ESR1;SPDEF;RARA;...  |
| FOXA2.A | FOXA1                   | FOXA1                      | FOXA1               | FOXA1                  | FOXA1;GATA3;ESR1;SPDEF;RARA;...  |
| FOXA3.B | FOXA1                   | FOXA1                      | FOXA1;FOXD2         | FOXA1                  | FOXA1;GATA3;ESR1;SPDEF;RARA;...  |

Table 13 – First twenty rows of the *getTF.hypo.significant.TFs.with.motif.summary.csv* file created by *get.TFs* function (suffix "\_HUMAN.H11MO" was removed from motifs names). First column shows the enriched motif, "top\_5percent\_TFs" shows the top 5% TFs ranked (the same as all TFs to the left of the dashed line in figure 19), "potential.TFs.family" are the TF from the "top\_5percent" that belongs to the same family as the TF of the motif, "top.potential.TFs.family" is the highest ranked TF belonging to the same family as the TF of the motif (same as the first TF from "potential.TFs.family" column). The columns "potential.TFs.subfamily" and "top.potential.TFs.subfamily" are the same as "potential.TFs.family" and "top.potential.TFs.family" but considering the subfamily classification instead. For example, the motif ANDR has two TFs in the top 5% that belongs to the same TF family (Steroid hormone receptors): ESR1 and AR, but if considering subfamilies only AR is considered.

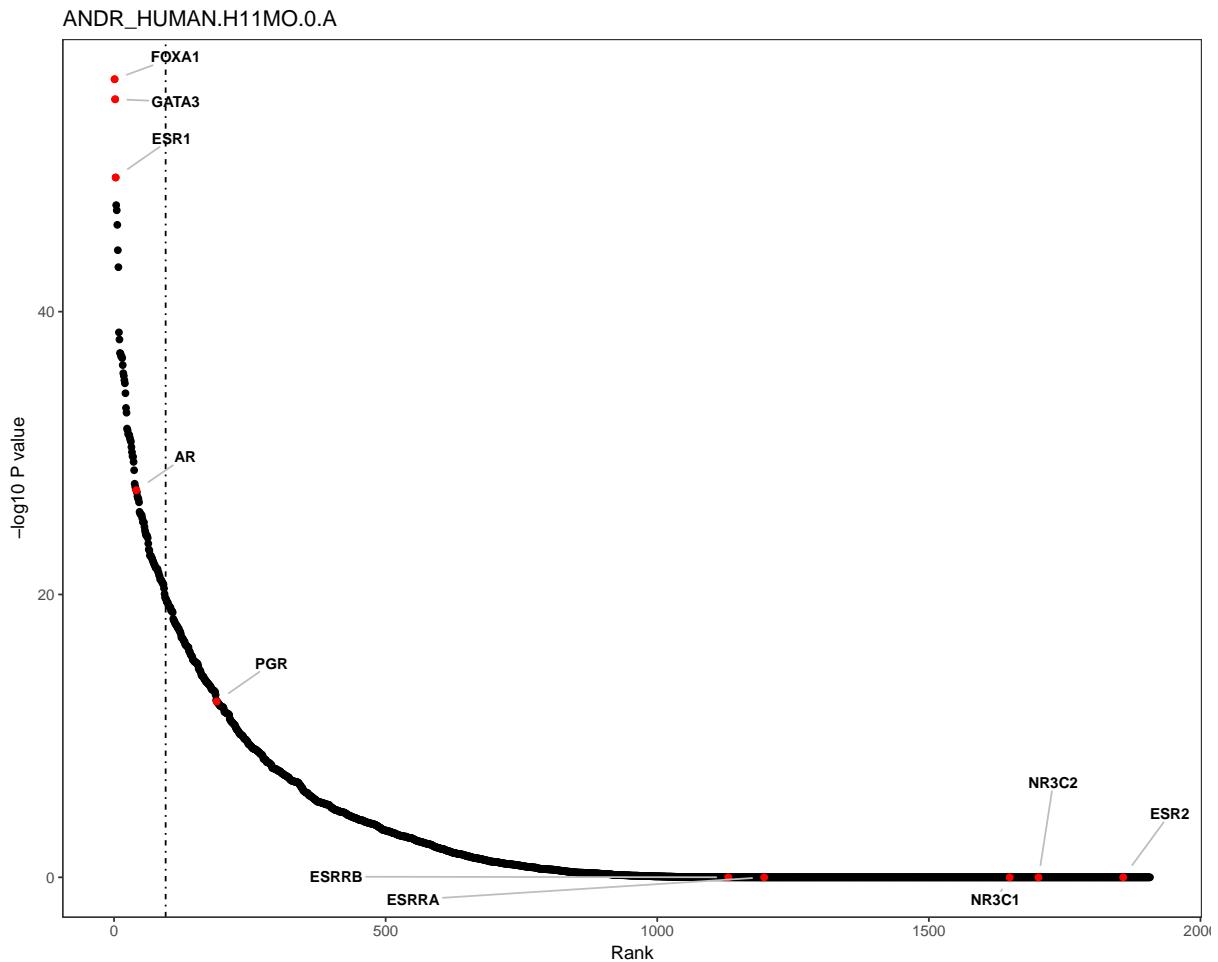


Figure 19 – TF ranking plot shows statistic  $-\log_{10}(P\text{-value})$  assessing the anti-correlation level of TFs expression level with average DNA methylation level at sites with a given motif. By default, the top 3 associated TFs and the TF family members (dots in red) that are associated with that specific motif are labeled in the plot. But there is also an option to highlight only TF sub-family members (TCClass database classification)

## Comparing inferred results with MCF-7 chIA-PET

As shown in Yao *et al.* (2015), we compared the putative pairs inferred to the chromatin loops derived from deep-sequenced ChIA-PET data from MCF7 cells (LI *et al.*, 2012). First, we identify the number of *ELMER* pairs overlapping the ChIA-PET loops, then we repeat using randomly generated pairs with properties similar to the *ELMER* pairs. For each true *ELMER* probe in a probe-gene pair, we randomly select a different probe from the complete set of distal probes. We then choose the nth nearest gene to the random probe, where n is the same as the adjacency of the true *ELMER* probe (i.e. if the true probe is linked to the second gene upstream, the random probe will also be linked to its second gene upstream). Thus, the random linkage set has both the same number of probes and the same number of linked genes as the true set. One hundred such random datasets were generated to arrive at a 95% CI ( $\pm 1.96 * SD$ ). The result is shown in Figure 20. Of the 2124 putative pairs identified in breast cancer tumors, 316 (approximately 14.9%) were also identified as loops in the MCF7 ChIA-PET data. This was a three-fold enrichment over randomized probe-gene pairs (see Additional file for the code).

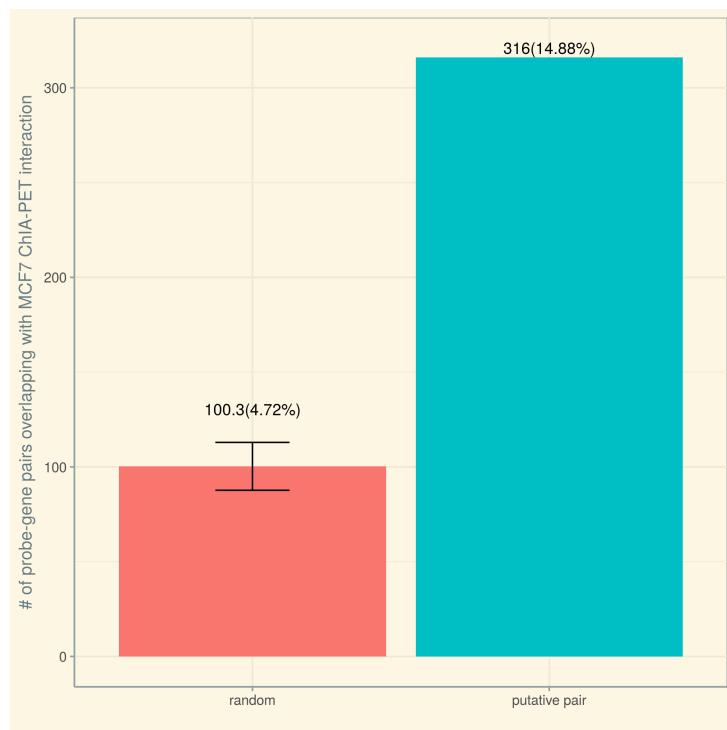


Figure 20 – The graph shows the comparison of the number of probe-gene pairs identified within MCF7 ChIA-PET data using the putative pairs from BRCA vs. random pairs

### 3.3.3 Use Case 2: BRCA molecular subtypes analysis (supervised approach)

Several studies identified distinct molecular Breast cancer classes and divided them as the luminal-like (Luminal A and Luminal B) subclasses, which are Estrogen receptor-positive (ER-positive), and the basal-like, ErbB2-positive and normal-like subclasses, which are the ER-negative groups (PEROU *et al.*, 2000; YERSAL; BARUTCA, 2014; SØRLIE *et al.*, 2001). To performed *ELMER* analysis comparing known molecular subtypes (Her2, Luminal A, Luminal B and Basal-like) a TCGA BRCA dataset classification retrieved from Ciriello *et al.* (2015).

The main arguments changed were the percentage of samples used to identify the differentially methylated probes in function *get.diff.meth* which was set to 100% (use all samples from each group) and the mode in function *get.pair* and in function *get.TFs* which was set to "supervised". In this mode instead of defining the U (unmethylated) group as the samples with lowest quintile of DNA methylation levels and the M (methylated) group as the highest quintile, the *U* and *M* group will be defined as all samples of one known molecular subtype (Figure ??). For example, if the first step identified probes hypomethylated in Luminal A group compared to Basa-like group, in the next steps the U group will be the Luminal A samples and the M group will be the Basal-like samples. The results and code of this supervised analysis can be found in the supplemental files.

From the Luminal A group vs Basal-like group analysis, it can be highlighted that the pairs of high expressed genes and hypomethylated probes in the Basal groups are enriched for the SOX10 TF signature. For this signature, the regulatory TF candidate identified is the SOX11 (Sry-related HMG box-11) TF; this correlation between basal-like and SOX11 was recently described by Shepherd *et al.* (2016). Also, the TF analysis reported the forkhead box C1 (FOXC1) transcription factor as the TF with the highest anti-correlation between TF expression and DNA methylation levels. Even though, this TF is not in the family of any enriched motifs this correlation between the FOXC1 TF and Basal-like breast cancers (BLBCs) was reported by Zuo e Yao (2016) and Chung *et al.* (2017).

On the other hand, when analyzing in the other direction in terms of methylation level, for those pairs of high expressed genes and hypomethylated probes in the Luminal A group, the TF analysis identified GATA3, FOXA1, RARA, MYB as candidate regulatory TF. GATA3 and FOXA1 are known characterize this molecular subtype as described by Yersal e Barutca (2014) while RARA was associated with the Luminal phenotype by Centritto *et al.* (2015), MYB is shown to be required for the proliferation of ER-positive breast cancer cells (MITRA *et al.*, 2016).

## Data availability

The TCGA data was downloaded from the NCI Genomic Data Commons (GDC) data portal (GROSSMAN *et al.*, 2016) using TCGAbiolinks R/Bioconductor package (COLAPRICO *et al.*, 2015; SILVA *et al.*, 2016). Gene annotations were retrieved from ENSEMBL (YATES *et al.*, 2015) database via biomaRt R/Bioconductor package (DURINCK *et al.*, 2005; DURINCK *et al.*, 2009). DNA methylation microarrays metadata were retrieved from <http://zwdzwd.github.io/InfiniumAnnotation> (ZHOU; LAIRD; SHEN, 2017). Transcription factor (TF) binding models can be downloaded at HOCOMOCO database (<http://hocomoco.autosome.ru/>) (KULAKOVSKIY *et al.*, 2016). The list of human TF can be accessed at <http://www.uniprot.org/> (APWEILER *et al.*, 2004). The classification of human transcription factors (TFs) can be viewed at <http://tfclass.bioinf.med.uni-goettingen.de/tfclass> (WINGENDER; SCHOEPS; DÖNITZ, 2013).

## Software availability

ELMER source code is available at <https://github.com/tiagochst/ELMER> and the auxiliary data files are available <https://github.com/tiagochst/ELMER.data>. *ELMER* is available under the GNU General Public License version 3 (GNU GPL3).

CHAPTER  
**4**

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## CANCER DATA ANALYSIS

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### 4.1 Glioma analysis

The Enhancer Linking by Methylation/Expression Relationship (ELMER) tool was used to analyze the molecular differences between the newly identified G-CIMP-low subtype of glioma that has some regions of the genome with a lower DNA methylation level and was associated with significantly worse survival compared to the G-CIMP-high, recently described by Dr. Noushmehr and his lab (CECCARELLI *et al.*, 2016b).

For this analysis, TCGA data from the NCI's Genomic Data Commons (GDC) was downloaded using our R/Bioconductor TCGAbiolinks package described in the previous chapter. Table 14 summarizes the number of samples in each group that have both deoxyribonucleic acid (DNA) methylation data for the Illumina HumanMethylation450 platform (HM450) and gene expression data (RNA-Seq) and table 15 summarizes the main values for the ELMER arguments. This analysis was performed with data aligned against the genome of reference hg38 and using ELMER "supervised" mode, which ensures that in all the steps the comparisons are made between each group using all their samples.

Table 14 – G-CIMP-high vs G-CIMP-low analysis: number of samples with both DNA methylation (HM450) and gene expression (RNA-seq) data.

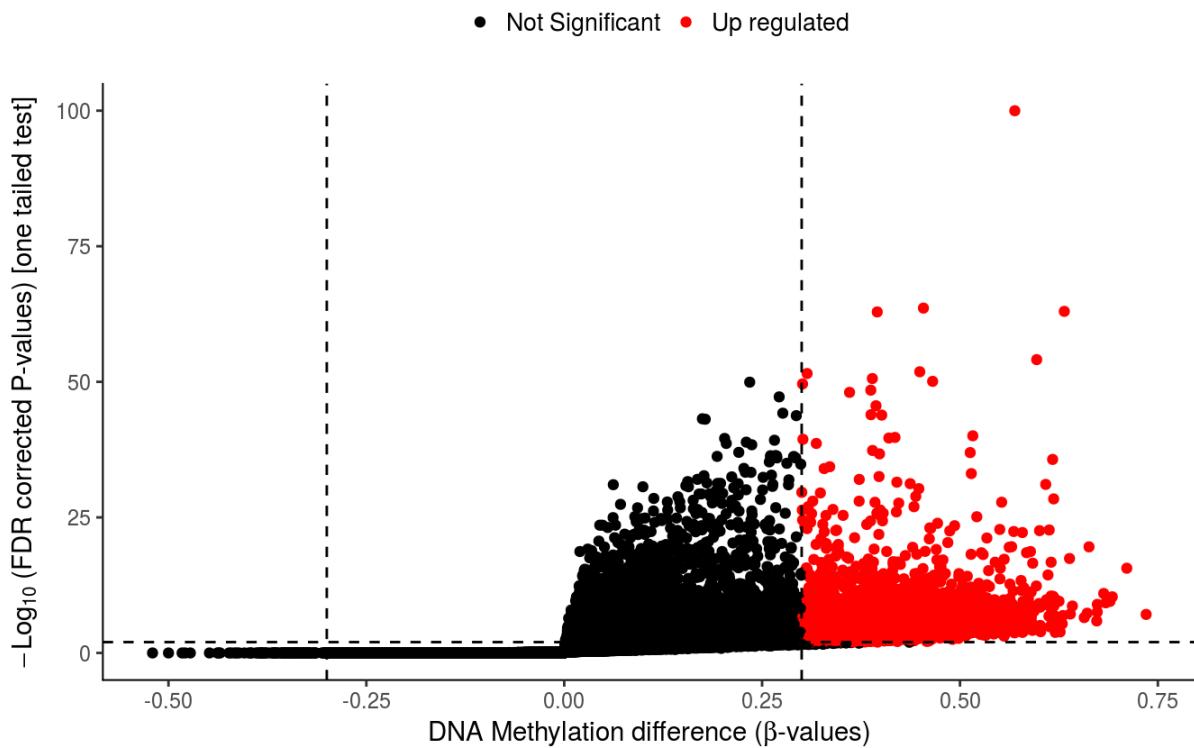
| Group       | Number of samples |
|-------------|-------------------|
| G-CIMP-high | 233               |
| G-CIMP-low  | 11                |

The DMCs (differently methylated CpGs) analysis, which uses a one-tailed t.test to find probes with higher mean level in GCIMP-high group compared to the GCIMP-low one. The result of this analysis is summarized in Figure 4.1. The volcano plot shows the difference of DNA methylation ( $\Delta\bar{\beta}$ ) versus significance  $-\log_{10}(\text{FDR corrected P-values})$ . Using as default cutt-offs  $\Delta\bar{\beta} \geq 0.3$  and  $-\log_{10}(\text{FDR corrected P-values}) \leq 0.01$ , 7540 probes were identified to be differently methylated.

Table 15 – G-CIMP-high vs G-CIMP-low analysis: ELMER arguments values.

| Step   | Argument                   | Value                  |
|--|----------------------------|------------------------|
| createMAE  | genome                     | hg38                   |
| Pairs probe-gene correlation/master regulator TF | Mode                       | Supervised             |
| All  | minSubgroupFrac            | 100%                   |
| All  | group1                     | GGimp-high             |
| All  | group2                     | GGimp-low              |
| All  | direction                  | hypermethylated probes |
| DMCs   | min $\Delta\bar{\beta}$    | 0.3                    |
| DMCs   | p-value adj cut-off        | 0.01                   |
| Pair probe-gene correlation                      | # permutations             | 10000                  |
| Pairs probe-gene correlation                     | raw p-value cut-off        | 0.001                  |
| Pairs probe-gene correlation                     | empirical p-values cut-off | 0.001                  |
| Motif enrichment                                 | minimum # probes           | 10                     |
| Motif enrichment                                 | lower OR                   | 1.1                    |

Volcano plot - Probes hypermethylated in gcimp-high vs g-cimp-low

Figure 21 – G-CIMP analysis: DNA methylation Volcano plot). Difference of DNA methylation (min $\Delta\bar{\beta}$ ) versus significance  $-\log_{10}(FDRcorrectedP - values)$ .

Those differently methylated were then paired with their 10 upstream and 10 downstream genes and an anti-correlation test which searches for genes highly expressed when the DNA

methylation levels decreases was performed. Using as cutt-offs  $-\log_{10}(\text{raw P-values}) \leq 0.001$  and  $-\log_{10}(\text{Permuted corrected P-values}) \leq 0.001$  for a 10000 permutation correction approach, 886 pairs of gene and probes were identified to be anti-correlated. The result of this analysis is summarized in Figure 4.1. The heatmap is composed of other three heatmaps, one heatmap for the DNA methylation levels, one heatmap for the gene expression levels and one with the distance between the gene and the probe.

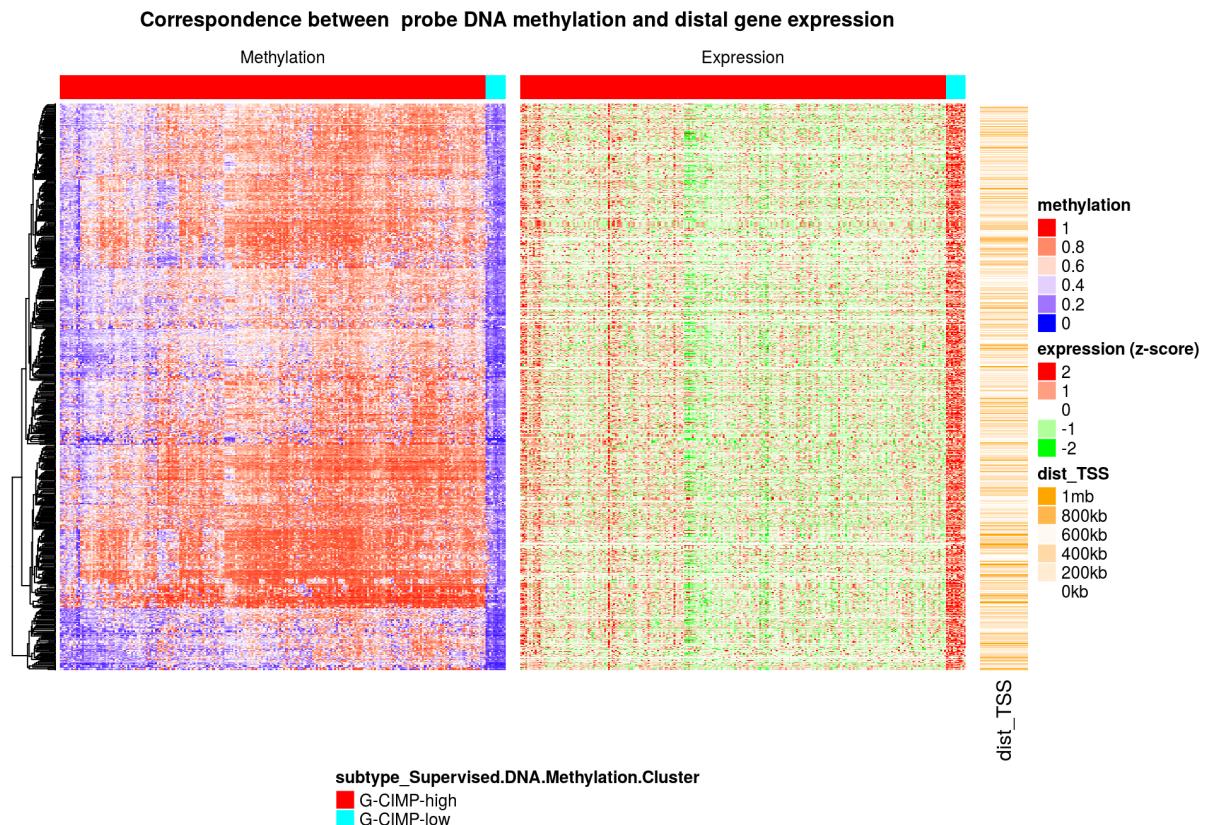


Figure 22 – G-CIMP analysis: Heatmap of paired probes and distal genes. The first heatmap (left one) shows DNA methylation  $\beta$  levels ranging from 0 (non-methylated) up to 1 (methylated probes). The second heatmap (middle one) shows z-scores for gene expression levels (standard deviations from each gene means). The last heatmap (right heatmap) shows the distance between the probe and gene anti-correlated.

Table 16 – G-CIMP-high vs G-CIMP-low analysis: number of distal probes, significant differently methylated probes, significant anti-correlated paired gene-probes and enriched motifs

| Result  | Value  |
|---|--------|
| Distal probes                                   | 135374 |
| Significant differently methylated probes       | 7540   |
| Siginificant anti-correlated paired gene-probes | 886    |
| Enriched motifs                                 | 84     |

The eriched motifs analysis results are summarized in Figure 4.1, which shows the Odds Ratio (x axis) for the enriched motifs, and in Table 17, which shows the candidate regulatory

TFs whose expression anti-correlated with the DNA methylation level on the probes of each enriched motifs. From the most anti-correlated ones, ELMER uses the TFClass classification (WINGENDER *et al.*, 2015) to identifies which TFs are known to bind in those motifs. This classification has two levels, family and subfamily, which groups motifs with a similar signature. Depending on the motif, its family classification might have a very similar signature, otherwise, the subfamily classification is the most indicated.

| Motif | Odds ratio<br>(95% CI) | # probes<br>(% of paired) |
|-------|------------------------|---------------------------|
| LBX2  | 2.11 (1.66–2.67)       | 78 (0.12%)                |
| NOTO  | 1.94 (1.56–2.41)       | 94 (0.14%)                |
| PDX1  | 1.87 (1.46–2.38)       | 73 (0.11%)                |
| HXD3  | 1.85 (1.46–2.35)       | 77 (0.12%)                |
| EMX2  | 1.81 (1.44–2.26)       | 89 (0.13%)                |
| SOX9  | 1.75 (1.44–2.12)       | 130 (0.19%)               |
| HXB3  | 1.74 (1.44–2.1)        | 135 (0.2%)                |
| HXA2  | 1.82 (1.44–2.29)       | 82 (0.12%)                |
| MIXL1 | 1.79 (1.43–2.25)       | 87 (0.13%)                |
| ISX   | 1.74 (1.42–2.13)       | 113 (0.17%)               |
| ZBT49 | 1.83 (1.41–2.38)       | 62 (0.09%)                |
| NFIA  | 1.74 (1.41–2.15)       | 100 (0.15%)               |
| P06F2 | 1.77 (1.41–2.23)       | 83 (0.12%)                |
| BATF  | 1.7 (1.4–2.07)         | 125 (0.19%)               |
| SOX13 | 1.72 (1.36–2.18)       | 78 (0.12%)                |
| SHOX  | 1.63 (1.35–1.98)       | 132 (0.2%)                |
| FOS   | 1.65 (1.35–2.01)       | 118 (0.18%)               |
| LHX6  | 1.66 (1.35–2.05)       | 104 (0.16%)               |
| GBX1  | 1.63 (1.34–1.98)       | 121 (0.18%)               |
| NOBOX | 1.69 (1.33–2.16)       | 74 (0.11%)                |
| DLX3  | 1.68 (1.32–2.13)       | 77 (0.12%)                |
| SOX5  | 1.63 (1.31–2.04)       | 90 (0.13%)                |
| HME2  | 1.71 (1.31–2.24)       | 59 (0.09%)                |
| IRF5  | 1.52 (1.3–1.78)        | 265 (0.4%)                |

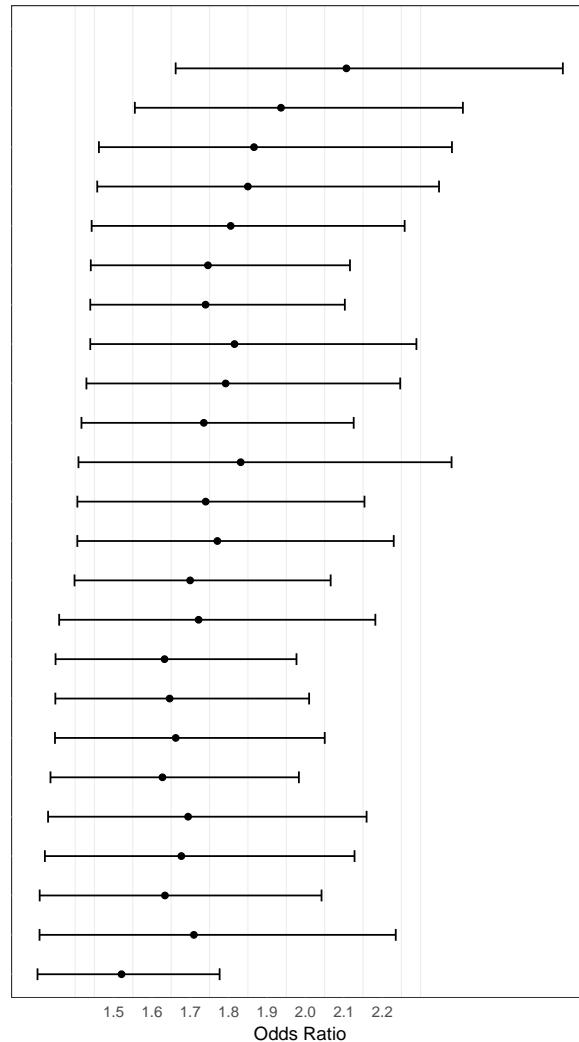


Figure 23 – Motif enrichment analysis: Odds Ratio (x axis) for the selected motifs with Odds Ratio (OR) above 1.5. The range shows the 95% confidence interval for each Odds Ratio.

For example, in Table 17 the motif HXD3 has as potential TF candidate the HOXD13 if we consider the family Transcription Factor (TF) classification and the HOXD3 TF candidate considering the subfamily TF classification. Figure 4.1 shows the motif signature for the HOX-related factors family from HOmo sapiens COmprehensive MOdel COllection (HOCOMOCO)

database. The transcription factors Homeobox D13 (HOXD13) and Homeobox D3 (HOXD3) are in the same family (HOX-related factors) but in different subfamilies.

Table 17 – TF ranking analysis: statistic For each enriched motif the anti-correlation level of all human TFs expression level with average DNA methylation level at sites with a given motif was access and ranked by the  $-\log_{10}(P_{value})$ , the most relevant one that belongs to the same family as the motif is shown in column *top.potential.TF.family* while the most relevant within the same sub-family classification is shown in column *top.potential.TF.subfamily*

| motif               | top.potential.TF.family | top.potential.TF.subfamily |
|---------------------|-------------------------|----------------------------|
| BATF_HUMAN.H10MO.A  | NA                      | NA                         |
| BATF_HUMAN.H10MO.S  | NA                      | NA                         |
| DLX3_HUMAN.H10MO.C  | HMX3                    | NA                         |
| EMX2_HUMAN.H10MO.D  | HMX3                    | NA                         |
| HME2_HUMAN.H10MO.D  | HMX3                    | NA                         |
| HXA2_HUMAN.H10MO.D  | HOXD13                  | HOXA2                      |
| HXB3_HUMAN.H10MO.D  | HOXD13                  | HOXD3                      |
| HXD3_HUMAN.H10MO.D  | HOXD13                  | HOXD3                      |
| IRF5_HUMAN.H10MO.D  | NA                      | NA                         |
| ISX_HUMAN.H10MO.D   | NA                      | NA                         |
| LBX2_HUMAN.H10MO.D  | HMX3                    | NA                         |
| MIXL1_HUMAN.H10MO.D | NA                      | NA                         |
| NOBOX_HUMAN.H10MO.C | NA                      | NA                         |
| NOTO_HUMAN.H10MO.D  | HMX3                    | NA                         |
| PDX1_HUMAN.H10MO.C  | HOXD13                  | NA                         |
| SHOX_HUMAN.H10MO.D  | NA                      | NA                         |
| SOX5_HUMAN.H10MO.C  | SOX11                   | NA                         |
| SOX9_HUMAN.H10MO.B  | SOX11                   | NA                         |
| ZBT49_HUMAN.H10MO.D | ZSCAN16                 | ZSCAN16                    |

To validate those findings, biological experiments are needed which by either knocking down the TF or by regulating the DNA methylation levels of those binding regions will be able to verify if the downstream genes are being regulated.

| Model                 | LOGO | Transcription factor  | Quality ⓘ | TF family                  | TF subfamily     |
|-----------------------|------|-----------------------|-----------|----------------------------|------------------|
|                       |      |                       | D         | HOX-related                |                  |
| HXD3_HUMAN.H11MO.0.D  |      | HOXD3<br>(GeneCards)  | D         | HOX-related factors[3.1.1] | HOX3[3.1.1.3]    |
| HXD4_HUMAN.H11MO.0.D  |      | HOXD4<br>(GeneCards)  | D         | HOX-related factors[3.1.1] | HOX4[3.1.1.4]    |
| HXA5_HUMAN.H11MO.0.D  |      | HOXA5<br>(GeneCards)  | D         | HOX-related factors[3.1.1] | HOX5[3.1.1.5]    |
| HXA7_HUMAN.H11MO.0.D  |      | HOXA7<br>(GeneCards)  | D         | HOX-related factors[3.1.1] | HOX6-7[3.1.1.6]  |
| HXB6_HUMAN.H11MO.0.D  |      | HOXB6<br>(GeneCards)  | D         | HOX-related factors[3.1.1] | HOX6-7[3.1.1.6]  |
| HXC6_HUMAN.H11MO.0.D  |      | HOXC6<br>(GeneCards)  | D         | HOX-related factors[3.1.1] | HOX6-7[3.1.1.6]  |
| HXC8_HUMAN.H11MO.0.D  |      | HOXC8<br>(GeneCards)  | D         | HOX-related factors[3.1.1] | HOX8[3.1.1.7]    |
| HXD8_HUMAN.H11MO.0.D  |      | HOXD8<br>(GeneCards)  | D         | HOX-related factors[3.1.1] | HOX8[3.1.1.7]    |
| HXA11_HUMAN.H11MO.0.D |      | HOXA11<br>(GeneCards) | D         | HOX-related factors[3.1.1] | HOX9-13[3.1.1.8] |
| HXC10_HUMAN.H11MO.0.D |      | HOXC10<br>(GeneCards) | D         | HOX-related factors[3.1.1] | HOX9-13[3.1.1.8] |
| HXC11_HUMAN.H11MO.0.D |      | HOXC11<br>(GeneCards) | D         | HOX-related factors[3.1.1] | HOX9-13[3.1.1.8] |
| HXC12_HUMAN.H11MO.0.D |      | HOXC12<br>(GeneCards) | D         | HOX-related factors[3.1.1] | HOX9-13[3.1.1.8] |
| HXC13_HUMAN.H11MO.0.D |      | HOXC13<br>(GeneCards) | D         | HOX-related factors[3.1.1] | HOX9-13[3.1.1.8] |
| HXD10_HUMAN.H11MO.0.D |      | HOXD10<br>(GeneCards) | D         | HOX-related factors[3.1.1] | HOX9-13[3.1.1.8] |
| HXD11_HUMAN.H11MO.0.D |      | HOXD11<br>(GeneCards) | D         | HOX-related factors[3.1.1] | HOX9-13[3.1.1.8] |
| HXD12_HUMAN.H11MO.0.D |      | HOXD12<br>(GeneCards) | D         | HOX-related factors[3.1.1] | HOX9-13[3.1.1.8] |
| HXD13_HUMAN.H11MO.0.D |      | HOXD13<br>(GeneCards) | D         | HOX-related factors[3.1.1] | HOX9-13[3.1.1.8] |

Figure 24 – HOCOMOCO V11: HOX-related factors family. Transcription factors HOXD13 and HOXD3 are in the same family (HOX-related factors) but in different subfamilies.



# CONCLUSION

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## 5.1 Conclusions

The main goal of this project has been to develop tools and workflows to perform integrative analysis as well as their application in the analysis of public cancer data. The motivations came from the fact that integrating data from different databases, stored in different formats and with different biological meanings is a complex process from both a computational point of view by dealing with big datasets which requires an optimized use of computational resources as well as from a biological point of view for dealing with a biological process still under study, often unknown, or even without a scientific consensus.

## 5.2 Conclusions and future studies

In this section, we list the conclusions and future studies for the results reported in the previous chapters.

### 5.2.1 *Conclusions and future works of TCGAbiolinks*

This work has presented an effort towards the search, download and prepare of data from the NCI's Genomic Data Commons (GDC) data portal for downstream analysis. It offered several single-dataset exploratory analysis such as PCA, clustering methods, box plots, differential gene expression analysis, differential methylated CpGs (DMCs) analysis, survival analysis and some integrative analysis such as the integration of differential methylated CpGs results with differential gene expression analysis to identify regions different methylated with the nearest gene level expression changed, and gene set enrichment analysis. As future works it is required the improvement of integrative analysis of the starburst plot, this method integrates the results from two different analysis DEA (differential expression analysis) and DMR (differential methylated regions). There is however a problem that the results are analyzed considering the set and not the same sample, that means the expression and methylation data of a sample are not compared to

each other. It is inferred if a data set has a differentiated average of methylation and a mean of expression of the nearest differentiated gene, and may even be different populations. This flaw is corrected in ELMER algorithms.

### **5.2.2 *Conclusions and future works of TCGAbiolinksGUI***

This work created a Graphical User Interface (GUI) to our command-line tools, with the purpose to help users without programming knowledge to perform deeper downstream analysis. Among possible improvements in future works are the export of figures in vector formats (PDF, SVG) that do not lose quality if altered (enlarged or reduced), the facilitation of the incorporation of external user data, which although can be incorporated if formatted in the current defined standard, this can still be improved, the creation of a modularization of the tool, which would load only the packages chosen by the user in order to decrease the amount of libraries needed to run the interface, which would be a challenge and may not be possible due to the limitations of the development tools used R / Shiny).

### **5.2.3 *Conclusions and future works of ELMER***

This work has presented an effort towards the integrative analysis performed using RNA-seq, DNA methylation and histone marks to identify a candidate regulatory network. It mainly identifies distal regions with a difference of DNA methylation and correlates them with the gene expression levels of upstream and downstream genes. A motif enrichment analysis is performed in the regulatory regions from the anti-correlated pairs (loss of DNA methylation and gain of gene expression) to identify potential regulatory TF candidates. As future works we suggest to expand the algorithm to the promoter regions, to expand the DNA methylation analysis to accept whole-genome bisulfite sequencing (WGBS) data, and to use mutation information instead of DNA methylation to identify regulatory regions mutated that might have affected the regulation of a upstream/downstream genes.

## **5.3 Publications, presentations and softwares of the Doctorate Period**

The work produced during the Doctorate period in form of scientific articles, softwares and conference presentations is shown in the next subsections. The published scientific articles were divided into two groups, one with the first authorship ones, and the other with the co-authorship ones. Those are listed in the following subsection: "First-authored papers", "Co-authored papers", "First-authored softwares" and "Co-authored softwares". The subsection "workshops and workflows" contains all the material created to help users to use the developed

tools. Finally, the subsection "Conferences & presentations" list all oral and poster presentations in international and national conferences.

### 5.3.1 First-authored papers

- COLAPRICO, A.; SILVA, T. C.; OLSEN, C.; GAROFANO, L.; CAVA, C.; GAROLINI, D.; SABEDOT, T. S.; MALTA, T. M.; PAGNOTTA, S. M.; CASTIGLIONI, I.; CECCARELLI, M.; BONTEMPI, G.; NOUSHMEHR, H. TCGAbiolinks: an r/bioconductor package for integrative analysis of tcga data. **Nucleic Acids Research**, v. 44, n. 8, p. e71, 2016. Disponível em: <http://nar.oxfordjournals.org/content/44/8/e71.abstract>.
- SILVA, T.; COLAPRICO, A.; OLSEN, C.; D'ANGELO, F.; BONTEMPI, G.; CECCARELLI, M.; NOUSHMEHR, H. TCGA workflow: Analyze cancer genomics and epigenomics data using bioconductor packages [version 2; referees: 1 approved, 1 approved with reservations]. **F1000Research**, v. 5, n. 1542, 2016.
- SILVA, T. C.; COLAPRICO, A.; OLSEN, C.; BONTEMPI, G.; CECCARELLI, M.; BERMAN, B. P.; NOUSHMEHR, H. Tcgabiolinksgui: A graphical user interface to analyze gdc cancer molecular and clinical data. **bioRxiv**, Cold Spring Harbor Labs Journals, 2017. Disponível em: <http://www.biorxiv.org/content/early/2017/08/17/147496>.
- SILVA, T. C.; COETZEE, S. G.; YAO, L.; HAZELETT, D. J.; NOUSHMEHR, H.; BERMAN, B. P. Enhancer linking by methylation/expression relationships with the r package elmer version 2. **bioRxiv**, Cold Spring Harbor Labs Journals, p. 148726, 2017.

### 5.3.2 Co-authored papers

- LIN, D.-C.; DINH, H. Q.; XIE, J.-J.; MAYAKONDA, A.; SILVA, T. C.; JIANG, Y.-Y.; DING, L.-W.; HE, J.-Z.; XU, X.-E.; HAO, J.-J.; WANG, M.-R.; LI, C.; XU, L.-Y.; LI, E.-M.; BERMAN, B. P.; KOEFFLER, H. P. Identification of distinct mutational patterns and new driver genes in oesophageal squamous cell carcinomas and adenocarcinomas. **Gut**, BMJ Publishing Group, 2017. ISSN 0017-5749. Disponível em: <http://gut.bmj.com/content/early/2017/09/02/gutjnl-2017-314607>.
- CECCARELLI, M.; BARTHEL, F.; MALTA, T.; SABEDOT, T.; SALAMA, S.; MURRAY, B.; AL., O. M. et. Molecular profiling reveals biologically discrete subsets and pathways of progression in diffuse glioma. **Cell**, v. 164, n. 3, p. 550 – 563, 2016. ISSN 0092-8674. Disponível em: <http://www.sciencedirect.com/science/article/pii/S009286741501692X>.
- MALTA, T. M.; SOUZA, C. F.; SABEDOT, T. S.; SILVA, T. C.; MOSELLA, M. Q.; KALKANIS, S. N.; SNYDER, J.; CASTRO, A. V. B.; NOUSHMEHR, H. Glioma cpg

island methylator phenotype (g-cimp): Biological and clinical implications. **bioRxiv**, Cold Spring Harbor Labs Journals, p. 169680, 2017.

- CAVA, C.; COLAPRICO, A.; BERTOLI, G.; GRAUDENZI, A.; SILVA, T. C.; OLSEN, C.; NOUSHMEHR, H.; BONTEMPI, G.; MAURI, G.; CASTIGLIONI, I. Spidermir: An r/bioconductor package for integrative analysis with mirna data. **International journal of molecular sciences**, Multidisciplinary Digital Publishing Institute, v. 18, n. 2, p. 274, 2017.

### 5.3.3 *First-authored softwares*

**TCGAbiolinks (version 2.0)** An R/Bioconductor package for integrative analysis of TCGA data. Published in Bioconductor <http://bioconductor.org/packages/TCGAbiolinks/>. Source code available in GitHub <https://github.com/BioinformaticsFMRP/TCGAbiolinks>.

**TCGAbiolinksGUI** A Graphical User Interface to analyze cancer genomics and epigenomics data. Published in GitHub <https://github.com/BioinformaticsFMRP/TCGAbiolinksGUI>.

**ELMER (version 2.0)** Enhancer Linking by Methylation/Expression Relationship (ELMER) is package to identify tumor-specific changes in DNA methylation within distal enhancers, and link these enhancers to downstream target genes. Published in Bioconductor <http://bioconductor.org/packages/ELMER/>. Source code available in GitHub <https://github.com/tiagochst/ELMER>.

### 5.3.4 *Co-authored softwares*

**SpidermiR: An R/Bioconductor package for integrative network analysis with miRNA data**  
Published in Bioconductor <http://bioconductor.org/packages/SpidermiR/>. Source code available in GitHub <https://github.com/claudiacava/SpidermiR>.

### 5.3.5 *Workshops and workflows*

**Workshop** Integrative analysis workshop with TCGAbiolinks and ELMER. Dana Farber Cancer Institute, Boston, MA. Link to workshop: <https://bioinformaticsfmrp.github.io/Bioc2017.TCGAbiolinks.ELMER/index.html>. Source code available in GitHub <https://github.com/BioinformaticsFMRP/Bioc2017.TCGAbiolinks.ELMER>.

**Workflow** TCGA Workflow: Analyze cancer genomics and epigenomics data using Bioconductor packages. Available at <https://www.bioconductor.org/help/workflows/TCGAWorkflow/>. Source code available in GitHub <https://github.com/BioinformaticsFMRP/TCGAWorkflow>.

### **5.3.6 Conferences & presentations**

**SNOLA 2016 - Update on Neuro-Oncology - Oral presentation - 14/19/2016** "EPIGENOMIC AND TRANSCRIPTOMIC ANALYSIS OF ADULT GLIOMA REVEALS CANDIDATE DRIVER TRANSCRIPTION FACTORS INVOLVED IN GLIOMA PROGRESSION." Windsor Barra Hotel, Rio de Janeiro - Brazil

**Chromatin and Epigenetics - Poster presentation - 5/5/2017** TCGAbiolinksGUI: A Graphical User Interface to analyze cancer genomics and epigenomics data. EMBL, Heidelberg, Germany

**Omics Seminar - Oral presentation - 06/06/2017** Enhancer Linking by Methylation/Expression Relationship: a case study using Breast Cancer. Cedars-Sinai Medical Center, Los Angeles, California.

**Bioc2017 - Oral presentation - 07/28/2017** Workshop: Integrative analysis workshop with TCGAbiolinks and ELMER. Dana Farber Cancer Institute, Boston, MA. Link to workshop: <https://bioinformaticsfmrp.github.io/Bioc2017.TCGAbiolinks.ELMER/index.html>

**From Single to Multiomics - Poster presentation - 11/13/2017** Enhancer Linking by Methylation/Expression Relationships with the R package (ELMER) version 2. EMBL, Heidelberg, Germany



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