

ELMER: An R/Bioconductor Tool Inferring Regulatory Element Landscapes and Transcription Factor Networks Using Methylomes

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1 Introduction

This document provides an introduction of the *ELMER*, which is designed to combine DNA methylation and gene expression data from human tissues to infer multi-level cis-regulatory networks. *ELMER* uses DNA methylation to identify enhancers, and correlates enhancer state with expression of nearby genes to identify one or more transcriptional targets. Transcription factor (TF) binding site analysis of enhancers is coupled with expression analysis of all TFs to infer upstream regulators. This package can be easily applied to TCGA public available cancer data sets and custom DNA methylation and gene expression data sets.

ELMER analyses have 5 main steps:

1. Identify distal enhancer probes on HM450K.
2. Identify distal enhancer probes with significantly different DNA methylation level in control group and experiment group.
3. Identify putative target genes for differentially methylated distal enhancer probes.
4. Identify enriched motifs for the distal enhancer probes which are significantly differentially methylated and linked to putative target gene.
5. Identify regulatory TFs whose expression associate with DNA methylation at motifs.

1.1 Installing and loading ELMER

To install this package, start R and enter

```
source("http://bioconductor.org/biocLite.R")
biocLite("ELMER")
```

2 Download example data

The following steps can be used to download the example data set for *ELMER*.

```
#Example file download from URL: https://dl.dropboxusercontent.com/u/61961845/ELMER.example.tar.gz
URL <- "https://dl.dropboxusercontent.com/u/61961845/ELMER.example.tar.gz"
download.file(URL,destfile = "ELMER.example.tar.gz",method= "curl")
untar("./ELMER.example.tar.gz")
library(ELMER)
```

3 Quick start: running TCGA example

TCGA.pipe is a function for easily downloading TCGA data and performing all the analyses in *ELMER*. For illustration purpose, we skip the downloading step. The user can use the getTCGA function to download TCGA data or use TCGA.pipe by including "download" in the analysis option.

The following command will do distal enhancer DNA methylation analysis and predict putative target genes, motif analysis and identify regulatory transcription factors.

```
TCGA.pipe("LUSC",wd="./ELMER.example",cores=detectCores()/2,permu.size=300,Pe=0.01,
analysis = c("distal.probes","diffMeth","pair","motif","TF.search"),
diff.dir="hypo",rm.chr=paste0("chr",c("X","Y")))
```

```

## #####
## Select distal enhancer probes
## #####

## #####
## Get differential DNA methylation loci
## #####

## ~~~ MEE.data:  initializer ~~~

## #####
## Predict pairs
## #####

## 'select()' returned 1:1 mapping between keys and columns
## ~~~ MEE.data:  initializer ~~~

## Identify putative probe-gene pair for hypomethylated probes
## Calculate empirical P value.
## 'select()' returned 1:1 mapping between keys and columns
## ~~~ MEE.data:  initializer ~~~

## #####
## Motif search
## #####

## Identify enriched motif for hypomethylated probes
## 13 motifs are enriched.

## #####
## Search responsible TFs
## #####

## ~~~ MEE.data:  initializer ~~~

## Identify regulatory TF for enriched motif in hypomethylated probes

```

4 Input data

MEE.data object is the input for all main functions of *ELMER*. MEE.data object can be generated by `fetch.mee` function. In order to perform all analyses in *ELMER*, MEE.data needs at least 4 files: a matrix of DNA methylation from HM450K platform for multiple samples; a matrix of gene expression for the same samples; a GRanges object containing the information for probes on HM450K such as names and coordinates; a gene annotation which is also a GRanges object. When TCGA data are used, the sample information will be automatically generated by `fetch.mee` function. However sample information should be provided when using custom data.

4.1 DNA methylation data

DNA methylation data feeding to *ELMER* should be a matrix of DNA methylation beta (β) value for samples (column) and probes (row) processed from row HM450K array data. If TCGA data were used, level 3 processed

data from TCGA website will be downloaded and automatically transformed to the matrix by *ELMER*. The level 3 TCGA DNA methylation data were calculated as $(M/(M+U))$, where M represents the methylated allele intensity and U the unmethylated allele intensity. Beta values range from 0 to 1, reflecting the fraction of methylated alleles at each CpG in the each tumor; beta values close to 0 indicates low levels of DNA methylation and beta values close to 1 indicates high levels of DNA methylation.

If user have raw HM450K data, these data can be processed by *Methylumi* or *minfi* generating DNA methylation beta (β) value for each CpG site and multiple samples. The `getBeta` function in *minfi* can be used to generate a matrix of DNA methylation beta (β) value to feed in *ELMER*. And we recommend to save this matrix as `meth.rda` since `fetch.mee` can read in files by specifying files' path which will help to reduce memory usage.

```
load("./ELMER.example/Result/LUSC/LUSC_meth_refined.rda")
Meth[1:10, 1:2]

##          TCGA-43-3394-11A-01D-1551-05 TCGA-43-3920-11B-01D-1551-05
## cg00045114          0.8190894          0.8073763
## cg00050294          0.8423084          0.8241138
## cg00066722          0.9101127          0.9162212
## cg00093522          0.8751903          0.8864599
## cg00107046          0.3326016          0.3445508
## cg00116430          0.6097183          0.5952469
## cg00152117          0.7074149          0.6439695
## cg00163018          0.5928909          0.8250584
## cg00173804          0.9162264          0.9303684
## cg00223046          0.7826863          0.7744760
```

4.2 Gene expression data

Gene expression data feeding to *ELMER* should be a matrix of gene expression values for samples (column) and genes (row). Gene expression value can be generated from different platforms: array or RNA-seq. The row data should be processed by other software to get gene or transcript level gene expression calls such as mapping by tophat, calling expression value by cufflink, RSEM or GenomeStudio for expression array. It is recommended to normalize expression data making gene expression comparable across samples such as quantile normalization. User can refer TCGA RNA-seq analysis pipeline to do generate comparable gene expression data. Then transform the gene expression values from each sample to the matrix for feeding into *ELMER*. If users want to use TCGA data, *ELMER* has functions to download the level 3 RNA-seq Hiseq data from TCGA website and transform the data to the matrix for feeding into *ELMER*. It is recommended to save this matrix as `RNA.rda` since `fetch.mee` can read in files by specifying the path of files which will help to reduce memory usage.

```
load("./ELMER.example/Result/LUSC/LUSC_RNA_refined.rda")
GeneExp[1:10, 1:2]

##          TCGA-22-5472-01A-01R-1635-07 TCGA-22-5489-01A-01R-1635-07
## ID126767          0.0000000          0.000000
## ID343066          0.4303923          0.000000
## ID26574           10.0817831          10.717673
## ID24              6.4462711          6.386644
## ID23456           8.5929182          9.333097
## ID5825            10.5578756          9.878333
## ID25              10.7233258          11.075515
## ID27              8.9761542          9.569239
## ID29777           9.6415206          9.353424
## ID80325           8.9840983          9.177624
```

4.3 Sample information

Sample information should be stored as a data.frame object containing sample ID, group labels (control and experiment). Sample ID and groups labels are required. Other information for each sample can be added to this data.frame object. When TCGA data were used, group labels (control and experiment) will be automatically generated by fetch.mee function by specifying option TCGA=TRUE.

```
mee <- fetch.mee(meth=Meth, exp=GeneExp, TCGA=TRUE)
## ~~~ MEE.data:  initializer ~~~
head(getSample(mee))
```

	ID	meth.ID	exp.ID
## TCGA-43-3394-11	TCGA-43-3394-11	TCGA-43-3394-11A-01D-1551-05	TCGA-43-3394-11A-01R-1758-07
## TCGA-56-8305-01	TCGA-56-8305-01	TCGA-56-8305-01A-11D-2294-05	TCGA-56-8305-01A-11R-2296-07
## TCGA-56-8307-01	TCGA-56-8307-01	TCGA-56-8307-01A-11D-2294-05	TCGA-56-8307-01A-11R-2296-07
## TCGA-56-8308-01	TCGA-56-8308-01	TCGA-56-8308-01A-11D-2294-05	TCGA-56-8308-01A-11R-2296-07
## TCGA-56-8309-01	TCGA-56-8309-01	TCGA-56-8309-01A-11D-2294-05	TCGA-56-8309-01A-11R-2296-07
## TCGA-58-8386-01	TCGA-58-8386-01	TCGA-58-8386-01A-11D-2294-05	TCGA-58-8386-01A-11R-2296-07
##	TN		
## TCGA-43-3394-11	Control		
## TCGA-56-8305-01	Experiment		
## TCGA-56-8307-01	Experiment		
## TCGA-56-8308-01	Experiment		
## TCGA-56-8309-01	Experiment		
## TCGA-58-8386-01	Experiment		

4.4 Probe information

Probe information should be stored as a GRanges object containing the coordinates of each probe on the DNA methylation array and names of each probe. The default probe information is for HM450K fetching from *IlluminaHumanMethylation450kanno.ilmn12.hg19*

```
probe <- getAnnotation(IlluminaHumanMethylation450kanno.ilmn12.hg19, what="Locations")
probe <- GRanges(seqnames=probe$chr,
                 ranges=IRanges(probe$pos,
                                width=1,
                                names=rownames(probe)),
                 strand=probe$strand,
                 name=rownames(probe))
mee <- fetch.mee(meth=Meth, exp=GeneExp, TCGA=TRUE, probeInfo=probe)
## ~~~ MEE.data:  initializer ~~~
getProbeInfo(mee)
```

	seqnames	ranges	strand	name
##	<Rle>	<IRanges>	<Rle>	<character>
##	cg00116430	chr1 [94188268, 94188268]	+	cg00116430
##	cg00889627	chr1 [1959630, 1959630]	-	cg00889627
##	cg01071265	chr1 [160952651, 160952651]	+	cg01071265
##	cg01074104	chr1 [41324394, 41324394]	-	cg01074104

```
##      cg01393939      chr1 [ 87803705, 87803705]      +      |      cg01393939
##      ...      ...      ...      ...      ...
##      cg27584013      chr1 [ 23012439, 23012439]      +      |      cg27584013
##      cg27589988      chr1 [215147891, 215147891]      +      |      cg27589988
##      cg27637706      chr1 [ 3472204, 3472204]      -      |      cg27637706
##      ch.1.131529R      chr1 [ 3283394, 3283394]      +      |      ch.1.131529R
##      ch.1.173213985R      chr1 [174947362, 174947362]      +      |      ch.1.173213985R
##      -----
##      seqinfo: 24 sequences from an unspecified genome; no seqlengths
```

4.5 Gene information

Gene information should be stored as a GRanges object containing coordinates of each gene, gene id, gene symbol and gene isoform id. The default gene information is the UCSC gene annotation fetching from *Homo.sapiens* by *ELMER* function: txs.

```
geneAnnot <- txs()

## 'select()' returned 1:1 mapping between keys and columns

## In TCGA expression data, geneIDs were used as the rowname for each row. However, numbers
## can't be the rownames, "ID" was added to each gene id functioning as the rowname.
## If your geneID is consistent with the rownames of the gene expression matrix, adding "ID"
## to each geneID can be skipped.
geneAnnot$GENEID <- paste0("ID",geneAnnot$GENEID)
geneInfo <- promoters(geneAnnot,upstream = 0, downstream = 0)
save(geneInfo,file="/ELMER.example/Result/LUSC/geneAnnot.rda")
mee <- fetch.mee(meth=Meth, exp=GeneExp, TCGA=TRUE, geneInfo=geneInfo)

## ~~~ MEE.data: initializer ~~~

getGeneInfo(mee)

## GRanges object with 14046 ranges and 3 metadata columns:
##      seqnames      ranges strand |      GENEID      TXNAME      SYMBOL
##      <Rle>      <IRanges> <Rle> | <character> <character> <character>
##      [1]      chr1 [ 69091, 69090]      +      |      ID79501      uc001aal.1      OR4F5
##      [2]      chr1 [322037, 322036]      +      |      ID100133331      uc009vjk.2      LOC100133331
##      [3]      chr1 [324288, 324287]      +      |      ID100133331      uc021oeh.1      LOC100133331
##      [4]      chr1 [367659, 367658]      +      |      ID729759      uc010nxu.2      OR4F29
##      [5]      chr1 [762971, 762970]      +      |      ID643837      uc031pjj.1      LINC01128
##      ...      ...      ...      ...      ...      ...      ...
##      [14042] chrUn_gl000223 [119731, 119730]      -      |      ID7574      uc031tgh.1      ZNF26
##      [14043] chrUn_gl000228 [ 76142, 76141]      +      |      ID22947      uc031tgj.1      DUX4L1
##      [14044] chrUn_gl000228 [ 82754, 82753]      +      |      ID22947      uc031tgl.1      DUX4L1
##      [14045] chrUn_gl000228 [105896, 105895]      +      |      ID22947      uc031tgq.1      DUX4L1
##      [14046] chrUn_gl000228 [109202, 109201]      +      |      ID22947      uc031tgr.1      DUX4L1
##      -----
##      seqinfo: 93 sequences (1 circular) from hg19 genome
```

4.6 MEE.data

MEE.data object is the input for multiple main functions of *ELMER*. MEE.data contains the above 5 components and making MEE.data object by fetch.mee function will keep each component consistent with each other. For example, although DNA methylation and gene expression matrixes have different rows (probe for DNA methylation and geneid for gene expression), the column (samples) order should be same in the two matrixes. fetch.mee function will keep them consistent when it generates the MEE.data object.

```
mee <- fetch.mee(meth=Meth, exp=GeneExp, TCGA=TRUE, probeInfo=probe, geneInfo=geneInfo)
## ~~~ MEE.data:  initializer ~~~

mee

## *** Class MEE.data, method show ***
## * meth
## num [1:1728, 1:234] 0.819 0.842 0.91 0.875 0.333 ...
## - attr(*, "dimnames")=List of 2
## ..$ : chr [1:1728] "cg00045114" "cg00050294" "cg00066722" "cg00093522" ...
## ..$ : chr [1:234] "TCGA-43-3394-11A-01D-1551-05" "TCGA-56-8305-01A-11D-2294-05" "TCGA-56-8307-01A-11D-2294-05" ...
## NULL
## * exp
## num [1:3884, 1:234] 0 0.214 10.048 5.007 8.63 ...
## - attr(*, "dimnames")=List of 2
## ..$ : chr [1:3884] "ID126767" "ID343066" "ID26574" "ID24" ...
## ..$ : chr [1:234] "TCGA-43-3394-11A-01R-1758-07" "TCGA-56-8305-01A-11R-2296-07" "TCGA-56-8307-01A-11R-2296-07" ...
## NULL
## * sample
## 'data.frame': 234 obs. of 4 variables:
## $ ID : chr "TCGA-43-3394-11" "TCGA-56-8305-01" "TCGA-56-8307-01" "TCGA-56-8308-01" ...
## $ meth.ID: chr "TCGA-43-3394-11A-01D-1551-05" "TCGA-56-8305-01A-11D-2294-05" "TCGA-56-8307-01A-11D-2294-05" ...
## $ exp.ID : chr "TCGA-43-3394-11A-01R-1758-07" "TCGA-56-8305-01A-11R-2296-07" "TCGA-56-8307-01A-11R-2296-07" ...
## $ TN : chr "Control" "Experiment" "Experiment" "Experiment" ...
## NULL
## * probeInfo
## GRanges object with 1728 ranges and 1 metadata column:
##
##           seqnames          ranges strand |           name
##           <Rle>           <IRanges> <Rle> | <character>
## cg00116430 chr1 [ 94188268, 94188268] + | cg00116430
## cg00889627 chr1 [ 1959630, 1959630] - | cg00889627
## cg01071265 chr1 [160952651, 160952651] + | cg01071265
## cg01074104 chr1 [ 41324394, 41324394] - | cg01074104
## cg01393939 chr1 [ 87803705, 87803705] + | cg01393939
## ...      ...      ...      ...      ...
## cg27584013 chr1 [ 23012439, 23012439] + | cg27584013
## cg27589988 chr1 [215147891, 215147891] + | cg27589988
## cg27637706 chr1 [ 3472204, 3472204] - | cg27637706
## ch.1.131529R chr1 [ 3283394, 3283394] + | ch.1.131529R
## ch.1.173213985R chr1 [174947362, 174947362] + | ch.1.173213985R
## -----
## seqinfo: 24 sequences from an unspecified genome; no seqlengths
## * geneInfo
## GRanges object with 14046 ranges and 3 metadata columns:
```

```
##          seqnames          ranges strand |          GENEID          TXNAME          SYMBOL
##          <Rle>          <IRanges> <Rle> | <character> <character> <character>
##      [1]          chr1 [ 69091,  69090]   + |      ID79501 uc001aal.1          OR4F5
##      [2]          chr1 [322037, 322036]   + | ID100133331 uc009vjk.2 LOC100133331
##      [3]          chr1 [324288, 324287]   + | ID100133331 uc021oeh.1 LOC100133331
##      [4]          chr1 [367659, 367658]   + |      ID729759 uc010nxu.2          OR4F29
##      [5]          chr1 [762971, 762970]   + |      ID643837 uc031pjj.1          LINC01128
##      ...          ...          ...          ...          ...          ...
## [14042] chrUn_gl000223 [119731, 119730]   - |      ID7574 uc031tgh.1          ZNF26
## [14043] chrUn_gl000228 [ 76142,  76141]   + |      ID22947 uc031tgj.1          DUX4L1
## [14044] chrUn_gl000228 [ 82754,  82753]   + |      ID22947 uc031tgl.1          DUX4L1
## [14045] chrUn_gl000228 [105896, 105895]   + |      ID22947 uc031tgq.1          DUX4L1
## [14046] chrUn_gl000228 [109202, 109201]   + |      ID22947 uc031tgr.1          DUX4L1
## -----
## seqinfo: 93 sequences (1 circular) from hg19 genome
## ***** End Print (MEE.data) *****
```

5 Illustration of ELMER analysis steps

The example data set is a subset of chromosome 1 data from TCGA LUSC. ELMER analysis have 5 main steps which are shown below individually. TCGA.pipe introduced above is a pipeline combining all 5 steps and producing all results and figures.

5.1 Selection of probes within biofeatures

This step is to select HM450K probes, which locate far from TSS (at least 2Kb away) and within distal enhancer regions. These probes are called distal enhancer probes. For distal enhancer regions, we constructed a comprehensive list of putative enhancers combining REMC, ENCODE and FANTOM5 data. Enhancers were identified using ChromHMM for 98 tissues or cell lines from REMC and ENCODE project and we used the union of genomic elements labeled as EnhG1, EnhG2, EnhA1 or EnhA2 (representing intergenic and intragenic active enhancers) in any of the 98 cell types, resulting in a total of 389,967 non-overlapping enhancer regions. Additionally, FANTOM5 enhancers (43,011) identified by eRNAs for 400 distinct cell types were added to this list. By default, this comprehensive list of putative enhancer and TSS annotated by GENCODE V15 and UCSC-gene will be used to select distal enhancer probes. But user can use their own TSS annotation or features such as H3K27ac ChIP-seq in a certain cell line.

```
#get distal enhancer probes that are 2kb away from TSS and overlap with REMC and FANTOM5
#enhancers on chromosome 1
Probe <- get.feature.probe(rm.chr=paste0("chr",c(2:22,"X","Y")))
save(Probe,file="./ELMER.example/Result/LUSC/probeInfo_feature_distal.rda")
```

5.2 Identifying differentially methylated probes

This step is to identify DNA methylation changes at distal enhancer probes which is carried out by function `get.diff.meth`.

For each enhancer probe, function first ranked experiment samples and control samples by their DNA methylation beta values. To identify hypomethylated probes, function compared the lower control quintile (20% of control

samples with the lowest methylation) to the lower experiment quintile (20% of experiment samples with the lowest methylation), using an unpaired one-tailed t-test. Only the lower quintiles were used because we did not expect all cases to be from a single molecular subtype, and we sought to identify methylation changes within cases from the same molecular subtype. 20% (i.e. a quintile) was picked as a cutoff to include high enough sample numbers to yield t-test p-values that could overcome multiple hypothesis correction, 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 `get.diff.meth` function in the *ELMER*, and should be tuned based on sample sizes in individual studies. The one tailed t-test was used to rule out the null hypothesis: $\mu_{\text{experiment}} \geq \mu_{\text{control}}$, where $\mu_{\text{experiment}}$ is the mean methylation within the lowest experiment quintile and μ_{control} is the mean within the lowest control quintile. Raw p-values were adjusted for multiple hypothesis testing using the Benjamini-Hochberg method, and probes were selected when they had adjusted p-value less than 0.01. For additional stringency, probes were only selected if the methylation difference: $\Delta = \mu_{\text{experiment}} - \mu_{\text{control}}$ was greater than 0.3. The same method was used to identify hypermethylated probes, except we used upper experiment quintile and upper control quintile, and chose the opposite tail in the t-test.

If `save` parameter of `get.diff.meth` is true, two cvs files will be saved. If false, a data frame with the same content as the second file mentioned below will be reported.

The first file contains all statistic results for all probes which were fed into the function. Based on this file, user can change different P value or `sig.dir` cutoff to select the significant results without redo the analysis.

The second file contains statistic results for the probes that pass the significant criteria (P value and `sig.dir`).

Both files contain four columns: probe, pvalue, ExperimentMinControl, adjust.p.

1. probe: the name of probes.
2. pvalue: the raw P value from t-test.
3. ExperimentMinControl: methylation difference Δ .
4. adjust.p: adjusted P value for t-test.

```
## fetch.mee can take path as input.
mee <- fetch.mee(meth="/ELMER.example/Result/LUSC/LUSC_meth_refined.rda",
  exp="/ELMER.example/Result/LUSC/LUSC_RNA_refined.rda", TCGA=TRUE,
  probeInfo="/ELMER.example/Result/LUSC/probeInfo_feature_distal.rda",
  geneInfo="/ELMER.example/Result/LUSC/geneAnnot.rda")

## ~~~ MEE.data:  initializer ~~~

sig.diff <- get.diff.meth(mee, cores=detectCores()/2, dir.out="/ELMER.example/Result/LUSC",
  diff.dir="hypo", pvalue = 0.01)

sig.diff[1:10,] ## significantly hypomethylated probes

##           probe           pvalue ExperimentMinControl    adjust.p
## cg00045114 cg00045114 7.307478e-13          -0.3499588 2.419463e-12
## cg00050294 cg00050294 4.440329e-09          -0.5000920 1.057951e-08
## cg00093522 cg00093522 1.143338e-23          -0.3596944 1.001147e-22
## cg00163018 cg00163018 2.240026e-21          -0.3838195 1.558083e-20
## cg00173804 cg00173804 2.767232e-18          -0.3744557 1.455328e-17
## cg00223046 cg00223046 8.142751e-11          -0.3313773 2.317862e-10
## cg00255699 cg00255699 6.386989e-13          -0.4308994 2.126941e-12
## cg00292636 cg00292636 6.368018e-34          -0.4833375 2.112468e-32
## cg00329272 cg00329272 6.864176e-39          -0.4355332 5.638431e-37
## cg00340127 cg00340127 3.056522e-18          -0.5857140 1.602584e-17
```

```
# get.diff.meth automatically save output files.
# getMethdiff.hypo.probes.csv contains statistics for all the probes.
# getMethdiff.hypo.probes.significant.csv contains only the significant probes which
# is the same with sig.diff
dir(path = "./ELMER.example/Result/LUSC", pattern = "getMethdiff")
## [1] "getMethdiff.hypo.probes.csv"          "getMethdiff.hypo.probes.significant.csv"
```

5.3 Identifying putative probe-gene pairs

This step is to link enhancer probes with methylation changes to target genes with expression changes and report the putative target gene for selected probes. This is carried out by function `get.pair`.

For each enhancer probe with differential methylation, the closest 10 upstream genes and the closest 10 downstream genes were tested for correlation between methylation of the probe and expression of the gene. To select these genes, the probe-gene distance was defined as the distance from the probe to a transcription start site specified by the UCSC gene annotation. Thus, exactly 20 statistical tests were performed for each probe, as follows. For each probe-gene pair, the samples (all experiment samples and control samples) were 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 consisted of the lowest methylation quintile (the 20% of samples with the lowest methylation.) The 20%ile cutoff is a configurable parameter in the `get.pair` Default is 20% as a balance, which would allow us to identify 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 each candidate probe-gene pair, the Mann-Whitney U test was used to test the null hypothesis that overall gene expression in group M was 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 probe-gene pair tested, the raw p-value P_r was corrected for multiple hypothesis using a permutation approach as follows (implemented in the `get.permu` function of the *ELMER* package). The gene in the pair was held constant, and x random methylation probes were used 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" (greater than 2kb from an annotated transcription start site), in order to make these null-model probes qualitatively similar to the probe being tested. An empirical p-value P_e value was calculated using the following formula (which introduces a pseudo-count of 1):

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

This step is the most time consuming step since it requires a large amount calculations for permutation. The greater the permutation time is, the longer it will take. It is recommended to use multiple cores for this step. Default permutation time is 1000 which may need 12 hrs by 4 cores. However 10,000 permutations is recommended if high confidence results are desired but it may cost 2 days.

If save paramter of `get.pair` function is true, two cvs files will be output. If save parameter is false, a data frame with the same context as the second file mentioned as below will be output.

The first file contains all statistic results for all probe-gene pairs. Based on this file, user can change different P value or sig.dir cutoff to select the significant results without redo the analysis.

The second file contains statistic results for the probes that pass the significant criteria (P_e).

Both files contain four columns: probe, GeneID, Symbol, Distance, Sides, Raw.p, P_e .

1. Probe: the name of probes.
2. GeneID and Symbol is for the genes which are linked to the probe.

3. Distance: the distance between the probe and the gene.
4. Sides: right (R) side or left (L) side of probe and the rank based on distance. For example, L3 means the gene is the number 3 closest gene from the left side of the probe.
5. Raw.p: P value from the Mann-Whitney U test for each pair.
6. Pe: the empirical P value for each pair.

```
### identify target gene for significantly hypomethylated probes.

Sig.probes <- read.csv("./ELMER.example/Result/LUSC/getMethdiff.hypo.probes.significant.csv",
                      stringsAsFactors=FALSE)[,1]
head(Sig.probes) # significantly hypomethylated probes
## [1] "cg00045114" "cg00050294" "cg00093522" "cg00163018" "cg00173804" "cg00223046"

## Collect nearby 20 genes for Sig.probes
nearGenes <- GetNearGenes(TRange=getProbeInfo(mee,probe=Sig.probes),
                          geneAnnot=getGeneInfo(mee),cores=detectCores()/2)

## Identify significant probe-gene pairs
Hypo.pair <- get.pair(mee=mee,probes=Sig.probes,nearGenes=nearGenes,
                     permu.dir="./ELMER.example/Result/LUSC/permu",permu.size=300,Pe = 0.01,
                     dir.out="./ELMER.example/Result/LUSC",cores=detectCores()/2,label= "hypo")

## Calculate empirical P value.
head(Hypo.pair) ## significant probe-gene pairs

##          Probe      GeneID      Symbol      Distance      Sides      Raw.p      Pe
## cg20701183.ID8543 cg20701183 ID8543      LM04          2563      L2 7.453984e-14 0.003322259
## cg19403323.ID255928 cg19403323 ID255928      SYT14          87458      R1 1.671937e-12 0.003322259
## cg12213388.ID84451 cg12213388 ID84451      KIAA1804          993548      L4 2.527644e-12 0.003322259
## cg26607897.ID55811 cg26607897 ID55811      ADCY10          292476      R4 4.593610e-12 0.003322259
## cg10574861.ID8543 cg10574861 ID8543      LM04           4715      L2 4.770162e-12 0.003322259
## cg26607897.ID23432 cg26607897 ID23432      GPR161          563308      R7 8.048248e-12 0.003322259

# get.pair automatically save output files.
# getPair.hypo.all.pairs.statistic.csv contains statistics for all the probe-gene pairs.
# getPair.hypo.pairs.significant.csv contains only the significant probes which is
# same with Hypo.pair.
dir(path = "./ELMER.example/Result/LUSC", pattern = "getPair")

## [1] "getPair.hypo.all.pairs.statistic.csv"
## [2] "getPair.hypo.pairs.significant.csv"
## [3] "getPair.hypo.pairs.significant.withmotif.csv"
```

5.4 Motif enrichment analysis on the selected probes

This step is to identify enriched motif in a set of probes which is carried out by function `get.enriched.motif`.

The build in data `Probes.motif` is generated using FIMO with a p-value $\leq 1e-4$ to scan a ± 100 bp region around each probe using Factorbook motif position weight matrices (PWMs) and Jasper core human motif PWMs generated from the R package `MotifDb`. For each probe set tested (i.e. the list of gene-linked hypomethylated probes in a given experiment group), a motif enrichment Odds Ratio and a 95% confidence interval were calculated

using following formulas:

$$p = \frac{a}{a+b}$$

$$P = \frac{c}{c+d}$$

$$\text{Odds Ratio} = \frac{\frac{p}{1-p}}{\frac{P}{1-P}}$$

$$SD = \sqrt{\frac{1}{a} + \frac{1}{b} + \frac{1}{c} + \frac{1}{d}}$$

$$\text{lower boundary of 95\% confidence interval} = \exp(\ln OR - SD)$$

where a is the number of probes within the selected probe set that contain 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 enhancer probe set. 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. As described in the text, Odds Ratios were also used for ranking candidate motifs.

There will be two results if save parameter of get.enriched.motif is true. When save is false, only second result mentioned below will be reported.

The first one is a csv file. This file contains the Odds Ratio and 95% confidence interval for these Odds Ratios which pass the significant cutoff (lower.OR and min.incidence). It contains 5 columns: motif, NumOfProbes, OR, lowerOR and upperOR.

1. motif: the name of motif.
2. NumOfProbes: the number of probes with this motif in the given set of probes corresponding to min.incidence option.
3. OR: the Odds Ratio.
4. lowerOR: the lower boundary of 95% confidence interval.
5. upperOR: the upper boundary of 95% confidence interval.

The second file is a rda file listing each enriched motif and the probes containing the motif.

```
### identify enriched motif for significantly hypomethylated probes which
##have putative target genes.

Sig.probes.paired <- read.csv("./ELMER.example/Result/LUSC/getPair.hypo.pairs.significant.csv",
                             stringsAsFactors=FALSE)[,1]
head(Sig.probes.paired) # significantly hypomethylated probes with putative target genes
## [1] "cg20701183" "cg19403323" "cg12213388" "cg26607897" "cg10574861" "cg26607897"

enriched.motif <-get.enriched.motif(probes=Sig.probes.paired,
                                   dir.out="./ELMER.example/Result/LUSC", label="hypo",
                                   min.incidence = 10,lower.OR = 1.1)

## 6 motifs are enriched.

names(enriched.motif) # enriched motifs
## [1] "AP1" "BARHL2" "IRF" "PRDM1" "TCF7L2" "TP53"

head(enriched.motif["TP53"]) ## probes in the given set that have TP53 motif.
```

```
## $TP53
## [1] "cg18437839" "cg11718886" "cg18437839" "cg22684969" "cg11718886" "cg06358191"
## [7] "cg11718886" "cg18161025" "cg11718886" "cg04923840" "cg24729839" "cg24296187"
## [13] "cg23972860" "cg18437839" "cg09858925" "cg06358191" "cg11718886" "cg06358191"
## [19] "cg11718886" "cg09858925" "cg18161025" "cg17181043" "cg22684969" "cg09858925"
## [25] "cg07347148" "cg06358191" "cg09874992" "cg11718886" "cg11958234" "cg17181043"
## [31] "cg00974095" "cg14420230" "cg11718886" "cg22684969" "cg18795022" "cg24296187"
## [37] "cg25001190" "cg07381872" "cg24296187" "cg02837162" "cg25001190" "cg24296187"
## [43] "cg20284239" "cg00935967" "cg11157208" "cg09814448" "cg11157208" "cg04105712"
## [49] "cg04105712"

# get.enriched.motif automatically save output files.
# getMotif.hypo.enriched.motifs.rda contains enriched motifs and the probes with the motif.
# getMotif.hypo.motif.enrichment.csv contains summary of enriched motifs.
dir(path = "./ELMER.example/Result/LUSC", pattern = "getMotif")

## [1] "getMotif.hypo.enriched.motifs.rda" "getMotif.hypo.motif.enrichment.csv"

# motif enrichment figure will be automatically generated.
dir(path = "./ELMER.example/Result/LUSC", pattern = "motif.enrichment.pdf")

## [1] "hypo.motif.enrichment.pdf"
```

5.5 Identifying regulatory TFs

This step is to identify regulatory TF whose expression associates with TF binding motif DNA methylation which is carried out by function `get.TFs`.

For each motif considered to be enriched within a particular probe set, function will compare the average DNA methylation at all distal enhancer probes within ± 100 bp of a motif occurrence, to the expression of 1,982 human TFs. A statistical test was performed for each motif-TF pair, as follows. The samples (all control and experiment samples) were divided into two groups: the M group, which consisted 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. The 20th percentile cutoff is a parameter to the `get.TFs` function and was set to allow for identification of molecular subtypes present in 20% of cases. For each candidate motif-TF pair, the Mann-Whitney U test was used to test the null hypothesis that overall gene expression in group M was 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 resulted in a raw p-value (Pr) for each of the 1982 TFs. All TFs were ranked by the $-\log_{10}(\text{Pr})$, and those falling within the top 5% of this ranking were considered candidate upstream regulators. The best upstream TFs which are known recognized the motif was automatically extracted as putative regulatory TFs.

If `save` parameter of `get.TFs` function is true, two files will be generated. If `save` parameter is false, only a data frame containing the same content with the first file mentioned below will be output.

The first one csv file. This file contain the regulatory TF significantly associate with average DNA methylation at particular motif sites. It contains 4 columns: motif, top.potential.TF, potential.TFs and top_5percent.

1. motif: the names of motif.
2. top.potential.TF: the highest ranking upstream TFs which are known recognized the motif.
3. potential.TFs: TFs which are within top 5% list and are known recognized the motif.
4. top_5percent: all TFs which are within top 5% list.

The second file is rda file. This file contains a matrix storing the statistic results for associations between TFs

(row) and average DNA methylation at motifs (column). This matrix can be use to generate TF ranking plots by function TF.rank.plot.

```
### identify regulatory TF for the enriched motifs

load("./ELMER.example/Result/LUSC/getMotif.hypo.enriched.motifs.rda")
TF <- get.TFs(mee=mee, enriched.motif=enriched.motif, dir.out="./ELMER.example/Result/LUSC",
             cores=detectCores()/2, label= "hypo")

# get.TFs automatically save output files.
# getTF.hypo.TFs.with.motif.pvalue.rda contains statistics for all TF with average
# DNA methylation at sites with the enriched motif.
# getTF.hypo.significant.TFs.with.motif.summary.csv contains only the significant probes.
dir(path = "./ELMER.example/Result/LUSC", pattern = "getTF")

## [1] "getTF.hypo.TFs.with.motif.pvalue.rda"
## [2] "getTF.hypo.significant.TFs.with.motif.summary.csv"

# TF ranking plot based on statistics will be automatically generated.
dir(path = "./ELMER.example/Result/LUSC/TFrankPlot", pattern = "pdf")

## [1] "BCL11A.TFrankPlot.pdf" "DMRT2.TFrankPlot.pdf" "FOXN1.TFrankPlot.pdf"
## [4] "MCM5.TFrankPlot.pdf" "NFE2L2.TFrankPlot.pdf" "OTX1.TFrankPlot.pdf"
## [7] "PIR.TFrankPlot.pdf" "PRICKLE3.TFrankPlot.pdf" "SMARCB1.TFrankPlot.pdf"
## [10] "SOX21.TFrankPlot.pdf" "TADA2A.TFrankPlot.pdf" "TP63.TFrankPlot.pdf"
## [13] "TTF2.TFrankPlot.pdf" "ZFP64.TFrankPlot.pdf"
```

6 Generating figures

6.1 Scatter plots

Generate scatter plots for one probes' nearby 20 gene expression vs DNA methylation at this probe. Figure 1

```
scatter.plot(mee,byProbe=list(probe=c("cg19403323"),geneNum=20),  
            category="TN", dir.out = "./ELMER.example/Result/LUSC", save=FALSE)  
## cg19403323
```

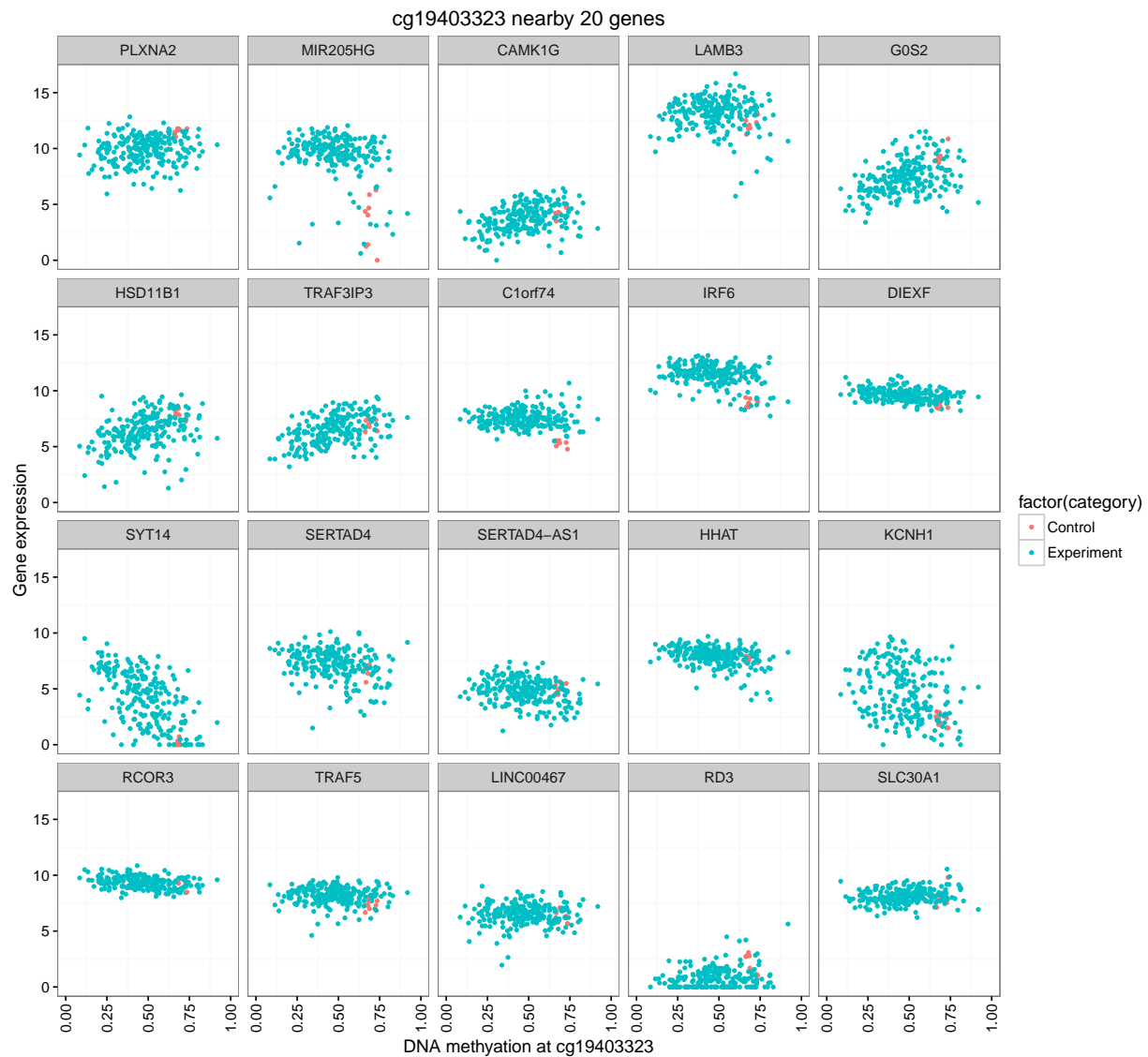


Figure 1: SEach scatter plot shows the methylation level of an example probe cg19403323 in all LUSC samples plotted against the expression of one of 20 adjacent genes.

6.1.1 Scatter plot of one pair

Generate a scatter plot for one probe-gene pair. Figure 2

```
scatter.plot(mee,byPair=list(probe=c("cg19403323"),gene=c("ID255928")),  
             category="TN", save=FALSE,lm_line=TRUE)
```

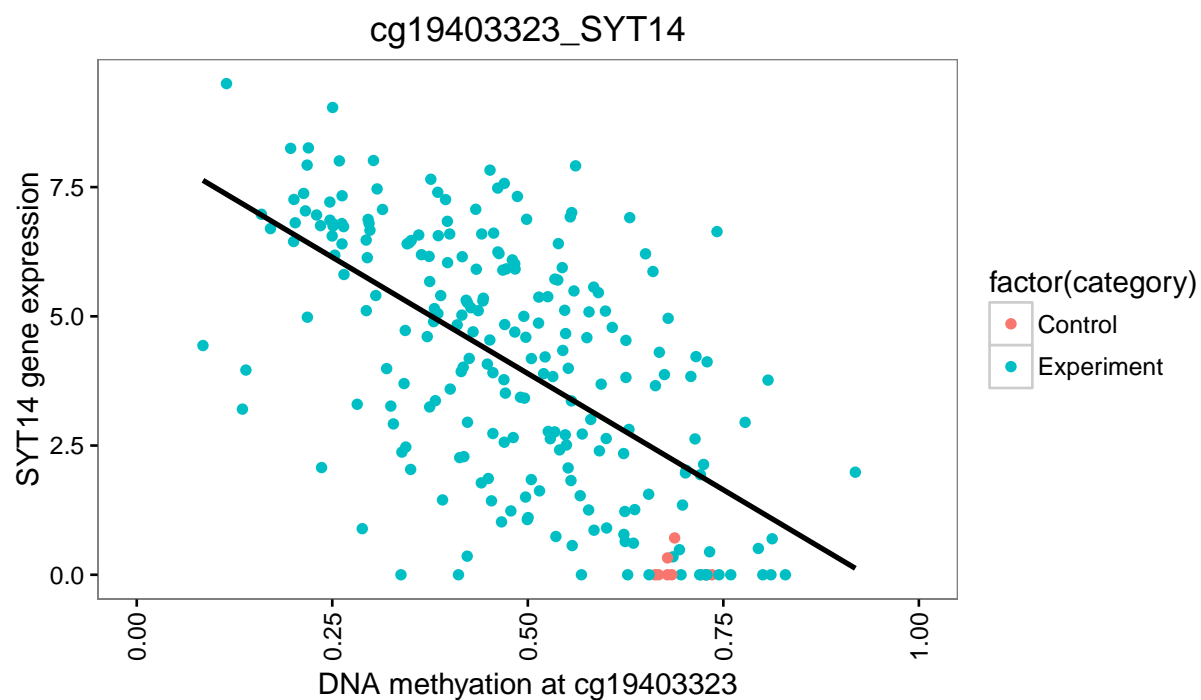


Figure 2: Scatter plot shows the methylation level of an example probe cg19403323 in all LUSC samples plotted against the expression of the putative target gene SYT14.

6.1.2 TF expression vs. average DNA methylation

Generate scatter plot for TF expression vs average DNA methylation of the sites with certain motif. Figure 3

```
load("ELMER.example/Result/LUSC/getMotif.hypo.enriched.motifs.rda")
scatter.plot(mee, byTF=list(TF=c("TP53", "TP63", "TP73"),
  probe=enriched.motif[["TP53"]]), category="TN",
  save=FALSE, lm_line=TRUE)
```

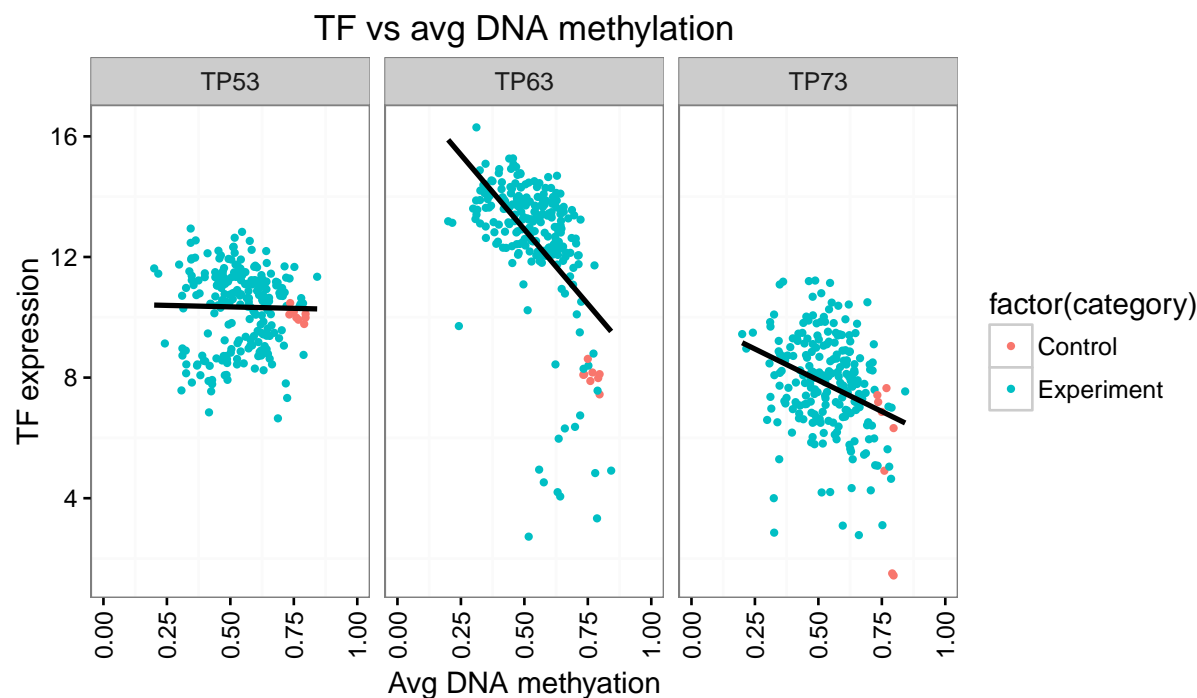


Figure 3: Each scatter plot shows the average methylation level of sites with the TP53 motif in all LUSC samples plotted against the expression of the transcription factor TP53, TP63, TP73 respectively.

6.2 Schematic plot

Schematic plot shows a brief view of linkages between genes and probes. To make a schematic plot, "Pair" object should be generated first.

```
# Make a "Pair" object for schematic.plot
pair <- fetch.pair(pair="./ELMER.example/Result/LUSC/getPair.hypo.pairs.significant.withmotif.csv",
  probeInfo = "./ELMER.example/Result/LUSC/probeInfo_feature_distal.rda",
  geneInfo = "./ELMER.example/Result/LUSC/geneAnnot.rda")

## ~~~ Pair:  initializer ~~~
```

6.2.1 Nearby Genes

Generate schematic plot for one probe with 20 nearby genes and label the gene significantly linked with the probe in red. Figure 4

```
schematic.plot(pair=pair, byProbe="cg19403323", save=TRUE)
## cg19403323
```

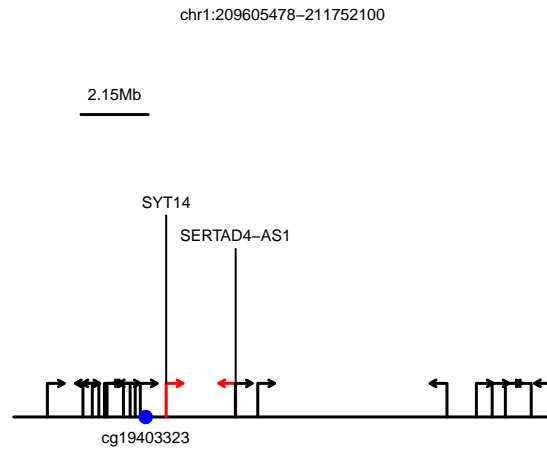


Figure 4: The schematic plot shows probe colored in blue and the location of nearby 20 genes. The genes significantly linked to the probe were shown in red.

6.2.2 Nearby Probes

Generate schematic plot for one gene with the probes which the gene is significantly linked to. Figure 5

```
schematic.plot(pair=pair, byGene="ID255928", save=TRUE)
```

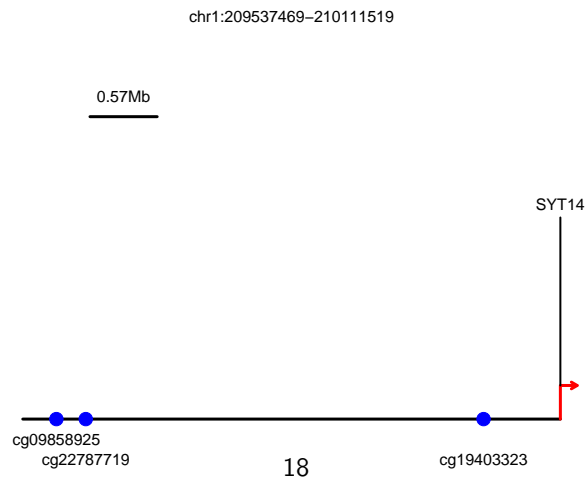


Figure 5: The schematic plot shows the gene colored in red and all blue-colored probes which are significantly

6.3 Motif enrichment plot

Motif enrichment plot shows the enrichment levels for the selected motifs. Figure 6

```
motif.enrichment.plot(motif.enrichment="./ELMER.example/Result/LUSC/getMotif.hypo.motif.enrichment.csv",
                     significant=list(OR=1.3,lowerOR=1.3), dir.out ="ELMER.example/Result/LUSC",
                     label="hypo", save=FALSE) ## different significant cut off.
```

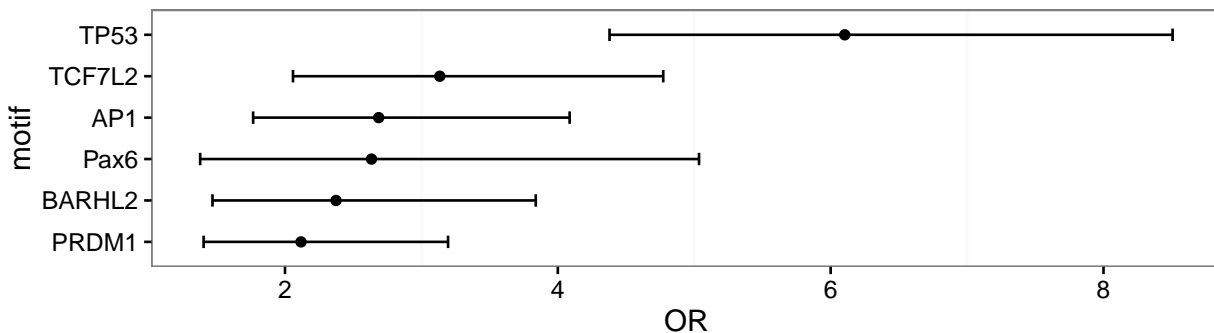


Figure 6: The plot shows the Odds Ratio (x axis) for the selected motifs with OR above 1.3 and lower boundary of OR above 1.3. The range shows the 95% confidence interval for each Odds Ratio.

6.4 TF ranking plot

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. Figure 7

```
load("./ELMER.example/Result/LUSC/getTF.hypo.TFs.with.motif.pvalue.rda")
TF.rank.plot(motif.pvalue=TF.meth.cor, motif="TP53", TF.label=list(TP53=c("TP53", "TP63", "TP73")),
             save=TRUE)
```

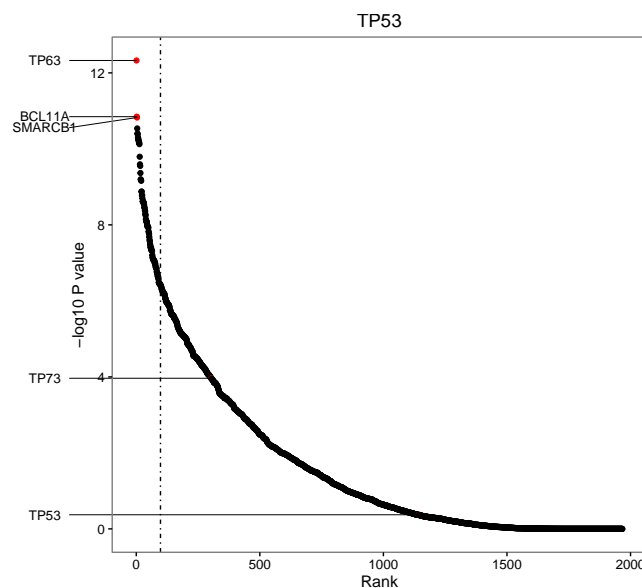


Figure 7: Shown are TF ranking plots based on the score ($-\log(P \text{ value})$) of association between TF expression and DNA methylation of the TP53 motif in the LUSC cancer type. The dashed line indicates the boundary of the top 5% association score. The top 3 associated TFs and the TF family members (dots in red) that are associated with that specific motif are labeled in the plot.

```

sessionInfo()

## R version 3.2.4 (2016-03-10)
## Platform: x86_64-w64-mingw32/x64 (64-bit)
## Running under: Windows Server 2008 R2 x64 (build 7601) Service Pack 1
##
## locale:
## [1] LC_COLLATE=C                      LC_CTYPE=English_United States.1252
## [3] LC_MONETARY=English_United States.1252 LC_NUMERIC=C
## [5] LC_TIME=English_United States.1252
##
## attached base packages:
## [1] stats4      parallel  stats      graphics  grDevices  utils      datasets  methods
## [9] base
##
## other attached packages:
## [1] ELMER_1.2.1
## [2] ELMER.data_1.0.0
## [3] Homo.sapiens_1.3.1
## [4] TxDb.Hsapiens.UCSC.hg19.knownGene_3.2.2
## [5] org.Hs.eg.db_3.2.3
## [6] GO.db_3.2.2
## [7] RSQLite_1.0.0
## [8] DBI_0.3.1
## [9] OrganismDbi_1.12.1
## [10] GenomicFeatures_1.22.13
## [11] AnnotationDbi_1.32.3
## [12] IlluminaHumanMethylation450kanno.ilmn12.hg19_0.2.1
## [13] minfi_1.16.1
## [14] bumpHunter_1.10.0
## [15] locfit_1.5-9.1
## [16] iterators_1.0.8
## [17] foreach_1.4.3
## [18] Biostrings_2.38.4
## [19] XVector_0.10.0
## [20] SummarizedExperiment_1.0.2
## [21] GenomicRanges_1.22.4
## [22] GenomeInfoDb_1.6.3
## [23] IRanges_2.4.8
## [24] S4Vectors_0.8.11
## [25] lattice_0.20-33
## [26] Biobase_2.30.0
## [27] BiocGenerics_0.16.1
##
## loaded via a namespace (and not attached):
## [1] nlme_3.1-126          bitops_1.0-6          matrixStats_0.50.1
## [4] RColorBrewer_1.1-2    tools_3.2.4           doRNG_1.6
## [7] norimix_1.2-1         colorspace_1.2-6      gridExtra_2.2.1
## [10] base64_1.1           preprocessCore_1.32.0  graph_1.48.0
## [13] formatR_1.3          pkgmaker_0.22         labeling_0.3
## [16] rtracklayer_1.30.4    scales_0.4.0          geneFilter_1.52.1

```

## [19] quadprog_1.5-5	RBGL_1.46.0	stringr_1.0.0
## [22] digest_0.6.9	Rsamtools_1.22.0	illuminaio_0.12.0
## [25] siggenes_1.44.0	GEOquery_2.36.0	limma_3.26.9
## [28] highr_0.5.1	BiocInstaller_1.20.1	mclust_5.2
## [31] BiocParallel_1.4.3	RCurl_1.95-4.8	magrittr_1.5
## [34] futile.logger_1.4.1	Rcpp_0.12.4	munsell_0.4.3
## [37] stringi_1.0-1	MASS_7.3-45	zlibbioc_1.16.0
## [40] plyr_1.8.3	grid_3.2.4	splines_3.2.4
## [43] multtest_2.26.0	annotate_1.48.0	knitr_1.12.3
## [46] beanplot_1.2	igraph_1.0.1	rngtools_1.2.4
## [49] corpcor_1.6.8	codetools_0.2-14	biomaRt_2.26.1
## [52] mixOmics_5.2.0	futile.options_1.0.0	XML_3.98-1.4
## [55] evaluate_0.8.3	lambda.r_1.1.7	gtable_0.2.0
## [58] reshape_0.8.5	ggplot2_2.1.0	xtable_1.8-2
## [61] survival_2.38-3	snow_0.4-1	GenomicAlignments_1.6.3
## [64] registry_0.3	ellipse_0.3-8	rgl_0.95.1441
## [67] BiocStyle_1.8.0		