# geneXtendeR

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## Introduction

This vignette describes `geneXtendeR` (Khomtchouk et al. 2016), an R/Bioconductor package for optimized annotation of genomic features (primarily peaks called from a ChIP-seq experiment, but any coverage island regions would work) with the nearest gene. "Extending" refers to performing gene-feature overlaps after adding to the gene-span a user-specified region upstream of the start of the gene model and a fixed (500 bp) region downstream of the gene, resulting in assigning to a gene the features that do not physically overlap with it but are sufficiently close. Extending is an automated iterative procedure in `geneXtendeR`, allowing the user to repeatedly align peaks to multiple gene transfer format (GTF) files to assess what global gene-spans optimize the genomewide alignment of peaks with their closest genes. This facilitates the process of deciphering which differentially enriched peaks are dysregulating which specific genes. This, in turn, aids experimental follow-up and validation in designing primers for a set of prospective genes during qPCR (Barbier et al. 2016).

### Rationale

With an abundance of Bioconductor software currently available for peak annotation to nearby features (e.g., `ChIPpeakAnno` (Zhu et al. 2010)) as well as the existence of various command line tools (e.g., `BEDTools` *closest* function (Quinlan and Hall, 2010)), what makes `geneXtendeR` different? The simple answer is: `geneXtendeR` is designed for assessing the variability of peak overlap with cis-regulatory elements and proximal-promoter regions. It is well-known that peak coordinates (peak start position, peak end position) exhibit a considerable degree of variance depending on the peak caller used (e.g., SICER (Zang et al. 2009), MACS2 (Zhang et al. 2008), etc.), both in terms of length distribution of peaks as well as the total number of peaks called, even when run at identical default parameter values (Koohy et al. 2014; Thomas et al. 2017). Tuning algorithm-specific parameters produces even greater

variance amongst peak callers, thereby complicating the issue further. This variance becomes a factor when annotating peak lists genome-wide with their nearest genes as, depending on the peak caller, peaks can be either shifted in genomic position (towards 5' or 3' end) or be of different lengths. As such, geneXtendeR represents a first step towards tailoring (or customizing) the functional annotation of a ChIP-seq peak dataset according to the details of the peak coordinates (chromosome number, peak start position, peak end position).

The primary focus of geneXtendeR is to optimize the process of functional annotation of a ChIP-seq peak list whereby instead of just annotating peaks with their nearest genomic features (as statically defined by a given genome build's coordinates), geneXtendeR investigates how peaks dynamically align to various user-specified gene extensions (e.g., 500 bp upstream extensions, 2000 bp upstream extensions, etc. for all genes in the genome). This shows where peaks localize across the genome with respect to their nearest gene, as well as what gene ontologies (BP, CC, and MF) are impacted at these various extension levels. This, in turn, informs the user what gene extensions ideally capture the GO terms involved in the biology of their experiment. For example, if a user's study is investigating the role of epigenetic enzymes in alcohol addiction and dependence, then functionally annotating a peak list using gene extensions that maximize the number of brain-related ontologies (for both BP, CC, and MF categories) makes sense.

With regards to histone modification ChIP-seq analysis, geneXtendeR computes optimal gene extensions tailored to the broadness of the specific epigenetic mark (e.g., H3K9me1, H3K27me3), as determined by a user-supplied ChIP-seq peak input file. To accomplish this level of custom-tailored data analysis, geneXtendeR first optimally extends the boundaries of every gene in a genome by some genomic distance (in DNA base pairs) for the purpose of flexibly incorporating cis-regulatory elements, such as promoter regions, as well as downstream elements that are important to the function of the gene relative to an epigenetic histone modification ChIP-seq dataset. This action effectively transforms genes into "gene-spheres", a new term that we coin to emphasize the 3D-nature of heterochromatin. A gene-sphere is composed of cis-regulatory elements (e.g., proximal promoters $+/- \approx 3$ kb from TSS), distal regulatory elements (e.g., enhancers), transcription start/end sites (TSS/TES), exons, introns, and downstream elements of a gene. As such, geneXtendeR maximizes the signal-to-noise ratio of locating genes closest to and directly under peaks. By performing a computational expansion of this nature, ChIP-seq reads that would initially not map strictly to a specific gene can now be optimally mapped to the regulatory regions of the gene, thereby implicating the gene as a potential candidate, and thereby making the ChIP-seq analysis more successful. Such an approach becomes particularly important when working with epigenetic histone modifications that have inherently broad peaks with a diffuse range of signal enrichment (e.g., H3K9me1, H3K27me3).

## Quick start

First, install the geneXtendeR package via:

```
> ## try http:// if https:// URLs are not supported
> source("https://bioconductor.org/biocLite.R")
> biocLite("geneXtendeR")
```

```
> library(geneXtendeR)
```

This automatically loads the `rtracklayer` R package, which contains the `readGFF()` command used to retrieve GTF files of any model organism. As such, load in a GTF file into your R environment, e.g.:

```
> rat <- readGFF("ftp://ftp.ensembl.org/pub/release-84/gtf/
+                        rattus_norvegicus/Rattus_norvegicus.Rnor_6.0.84.chr.gtf.gz")
```

URLs may be obtained as direct links from: http://useast.ensembl.org/info/data/ftp/index.html. Click on the "GTF" link under the "Gene sets" column for a particular species and then right-click (or command-click on Mac OS X) the name of the file containing the species name/version number and file extension `chr.gtf.gz` (e.g., Homo_sapiens.GRCh38.84.chr.gtf.gz, Mus_musculus.GRCm38.84.chr.gtf.gz, etc.), and copy the link address. Then, paste the link address into the `readGFF()` as shown above. This will create an R dataframe object containing the respective GTF file.

Next, the user must input their peak data from a peak caller (e.g., SICER, MACS2, etc.). The peak data must contain only three tab-delimited columns: chromosome number, peak start, and peak end. See `?samplepeaksinput` for an example. Once the peak input data (e.g., "somepeaksfile.txt") has been assembled properly (i.e., to contain only the three tab-delimited columns above), it must be properly formatted prior to the execution of downstream analyses.

First, the user must set their working directory to point to the location of their peak data file. Then type the following command:

```
> peaksInput("somepeaksfile.txt")
```

This command properly formats the user's peak file in preparation for subsequent analyses, producing a resultant "peaks.txt" file in the user's working directory.

To see how the above command works using a built-in example, the `geneXtendeR` package provides a peak input dataset[1] called "somepeaksfile.txt", which can be loaded into memory like this:

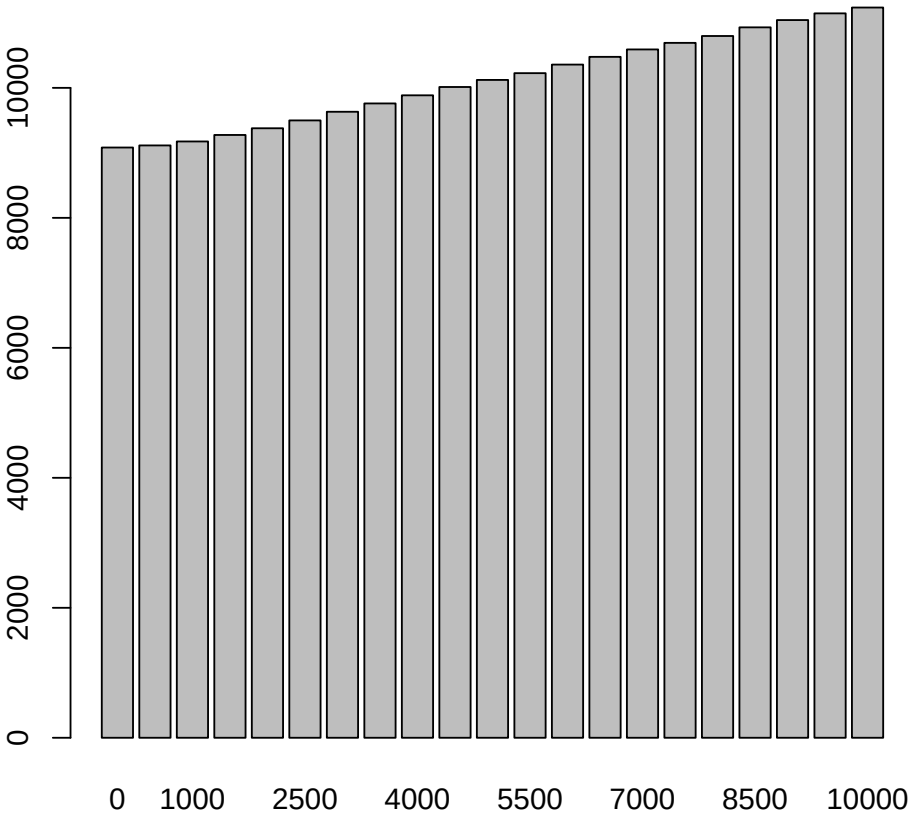
```
> fpath <- system.file("extdata", "somepeaksfile.txt", package="geneXtendeR")
> peaksInput(fpath)
```

This creates a properly formatted (i.e., properly sorted) "peaks.txt" file in the user's working directory.

Now, we may use the R object that we created with `readGFF()` earlier to create a bar chart visualization showing the number of peaks that are sitting directly on top of genes across a series of upstream extensions (of each gene in a genome):

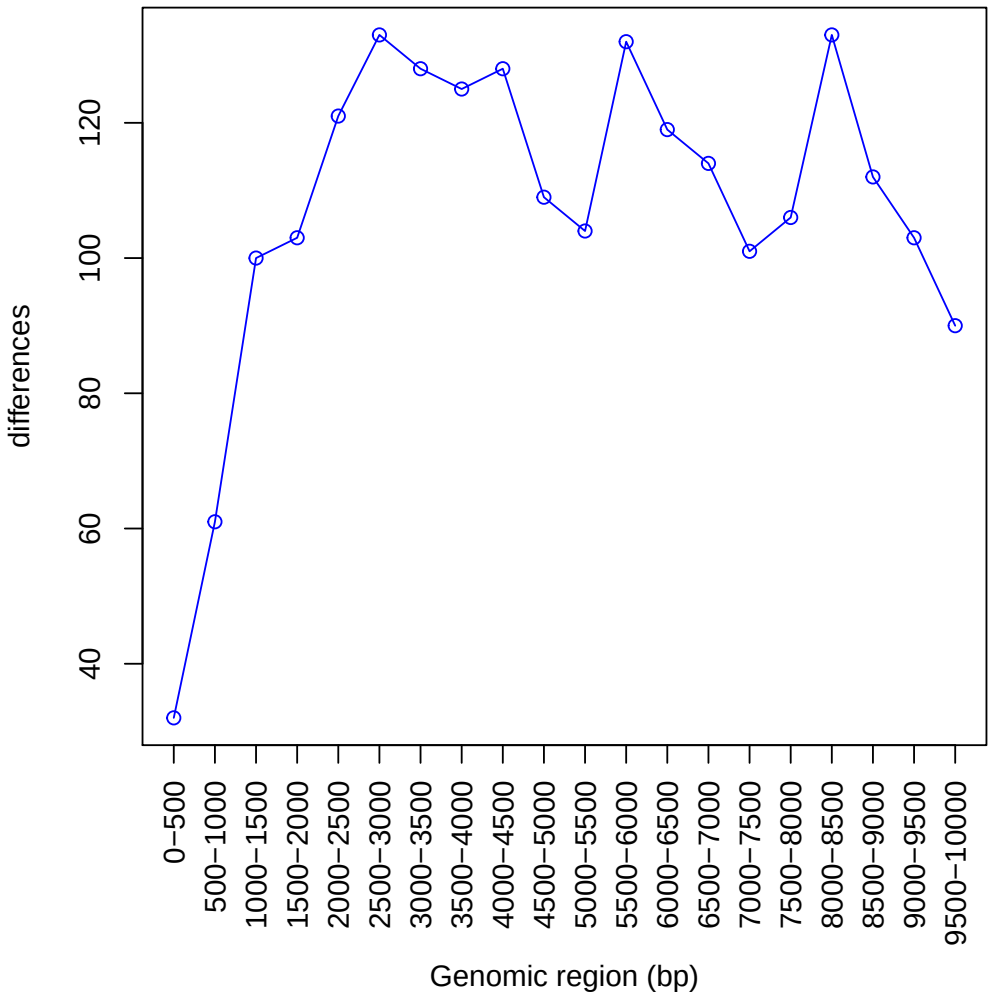
[1] This peaks dataset comes from a ChIP-seq investigation of brain tissue (prefrontal cortex) in alcohol addiction and dependence (Barbier et al. 2016), see References section for details.

```
> barChart(rat, 0, 10000, 500)
```



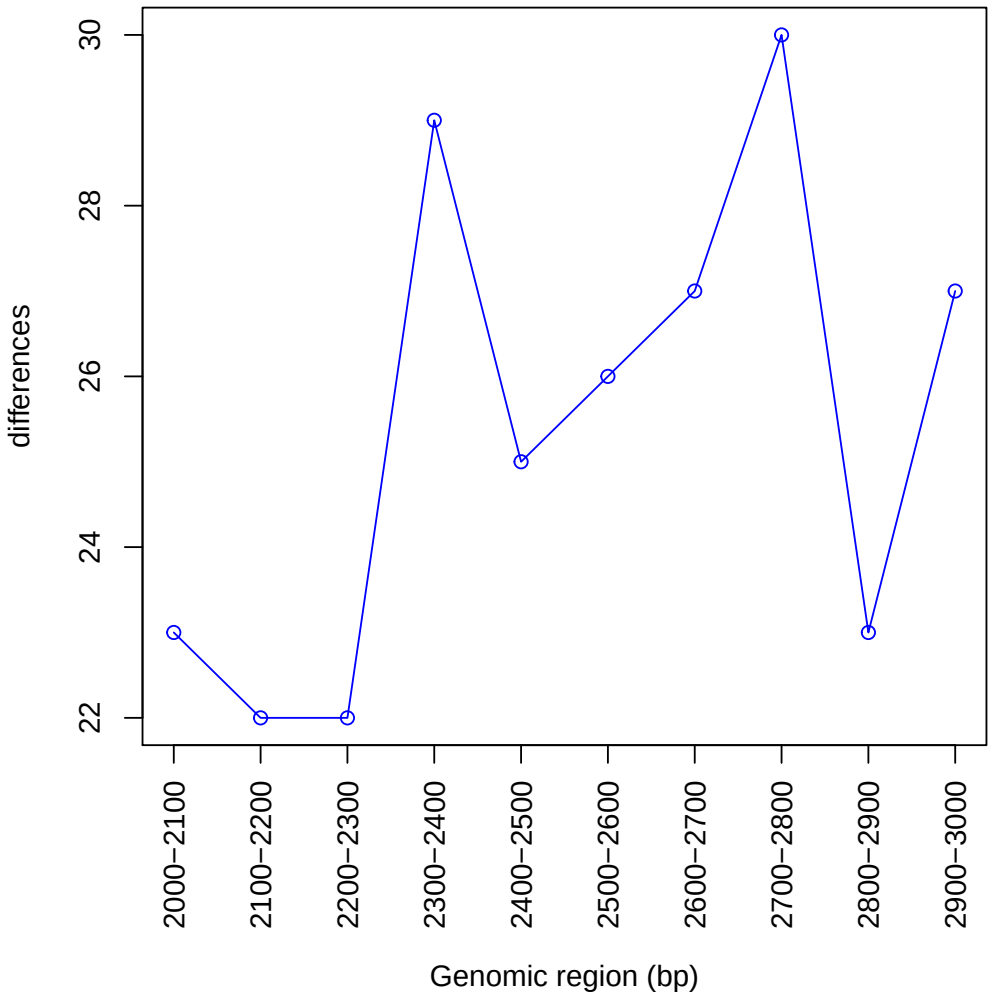

This command first generates 21 individual whole-genome files: 0, 500, 1000, ..., and 10000 bp upstream extension files for the rat (*Rattus norvegicus*) genome, each having an automatic 500 bp downstream extension. In other words, each gene in the rat genome is extended upstream and downstream by some user-specified distance, thereby creating a "gene-sphere." As such, this bar chart command visualizes the raw count of the number of peaks that are sitting on top of genes at each individual upstream cutoff. Clearly, the wider the gene-sphere, the more peaks-on-top-of-genes are found throughout the genome. However, the law of diminishing returns begins to kick in at increasing upstream extension levels (see `linePlot()` for a visual representation):

```
> linePlot(rat, 0, 10000, 500)
```



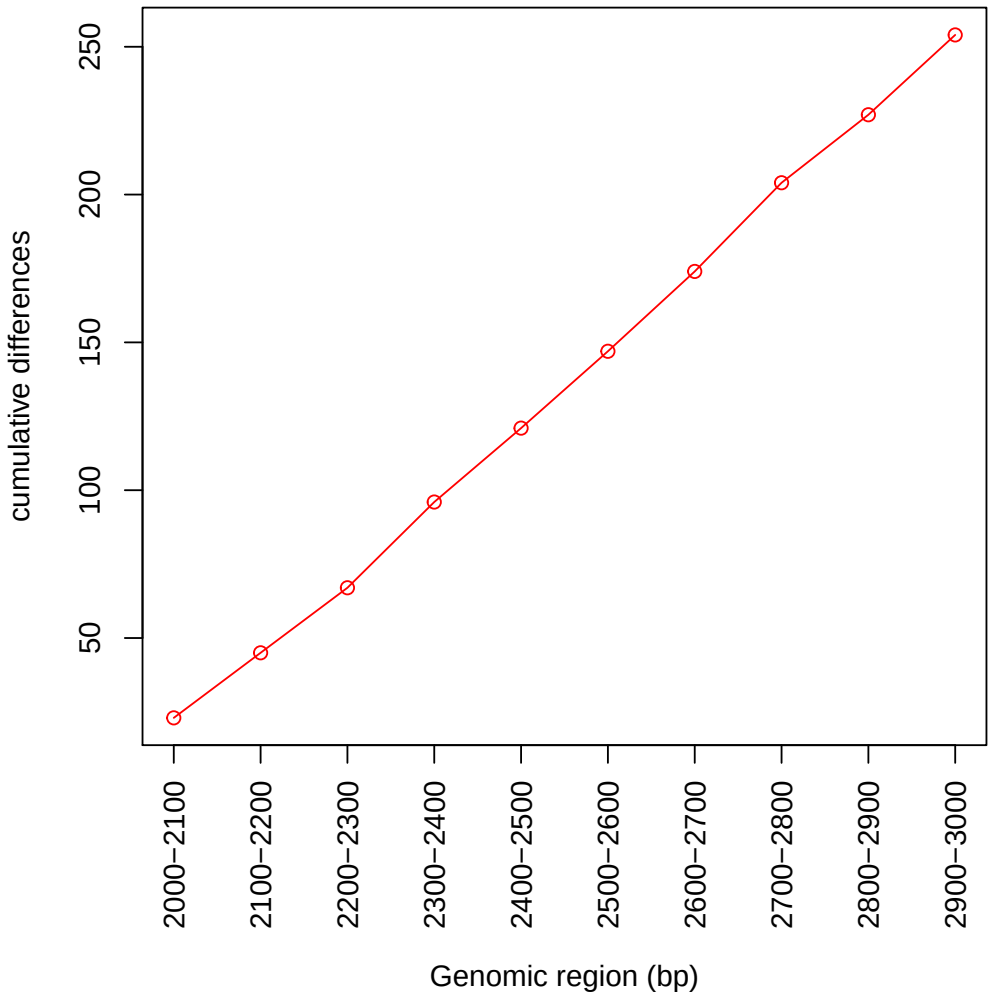

In this line plot, there is a sharp rise in the number of peaks-on-top-of-genes from a 0 bp upstream extension to a 1500 bp upstream extension, and from a 2000 bp upstream extension to a 3000 bp upstream extension. This steady rise up until 3000 bp is followed by a steady decline at subsequent extension levels followed by some noisy fluctuations. It may be interesting to investigate what is going on in the interval from 2000 bp to 3000 bp:

```
> linePlot(rat, 2000, 3000, 100)
```




Visually, there is a relative spike in the number of peaks-on-top-of-genes at the 2400 bp upstream extension (as compared to the 2300 bp extension). This spike then drops back down at subsequent extension levels and fluctuates in a noisy manner. However, a cumulative line plot shows that this "spike" is more of a visual effect than anything else, since the graph is almost perfectly linear:

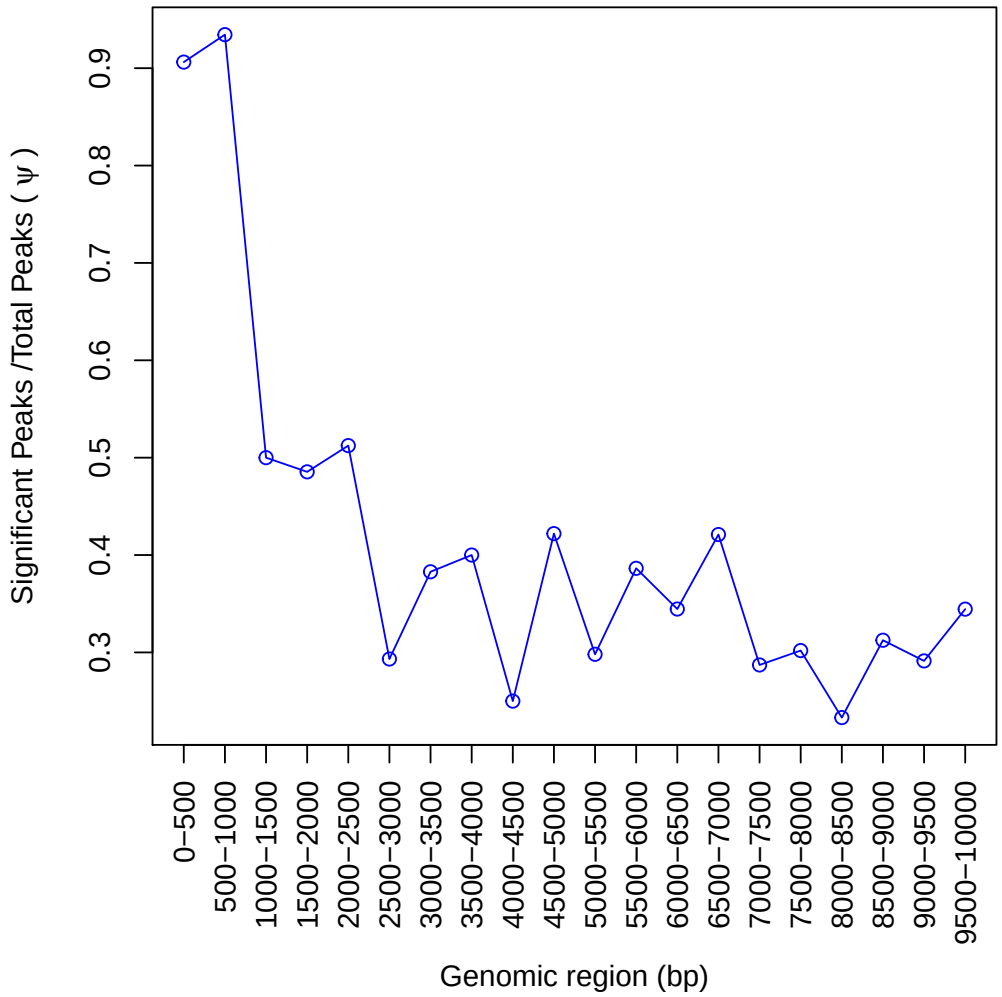
```
> cumlinePlot(rat, 2000, 3000, 100)
```




Hence, one very useful function in `geneXtendeR` is called `hotspotPlot()`, which allows users to examine the ratio of statistically significant peaks[2] to the total number of peaks at each genomic interval (e.g., 0-500 bp upstream of every gene in the genome, 500-1000 bp upstream of every gene in the genome, etc.).

[2]Note that statistical significance is set apriori by the user at the peak calling stage (prior to `geneXtendeR`) to give the user the freedom to choose how to filter out peak coordinates that only pass specific p-value and FDR cutoffs from a peak caller. Peak caller output (e.g., from SICER) gives both p-value and FDR measures for each peak, thereby making it easy to extract only the peak coordinates that pass a specific set of statistical cutoff criteria.

```
> allpeaks <- system.file("extdata", "totalpeaksfile.txt", package="geneXtendeR")
> sigpeaks <- system.file("extdata", "significantpeaksfile.txt", package="geneXtendeR")
> hotspotPlot(allpeaks, sigpeaks, rat, 0, 10000, 500)
```

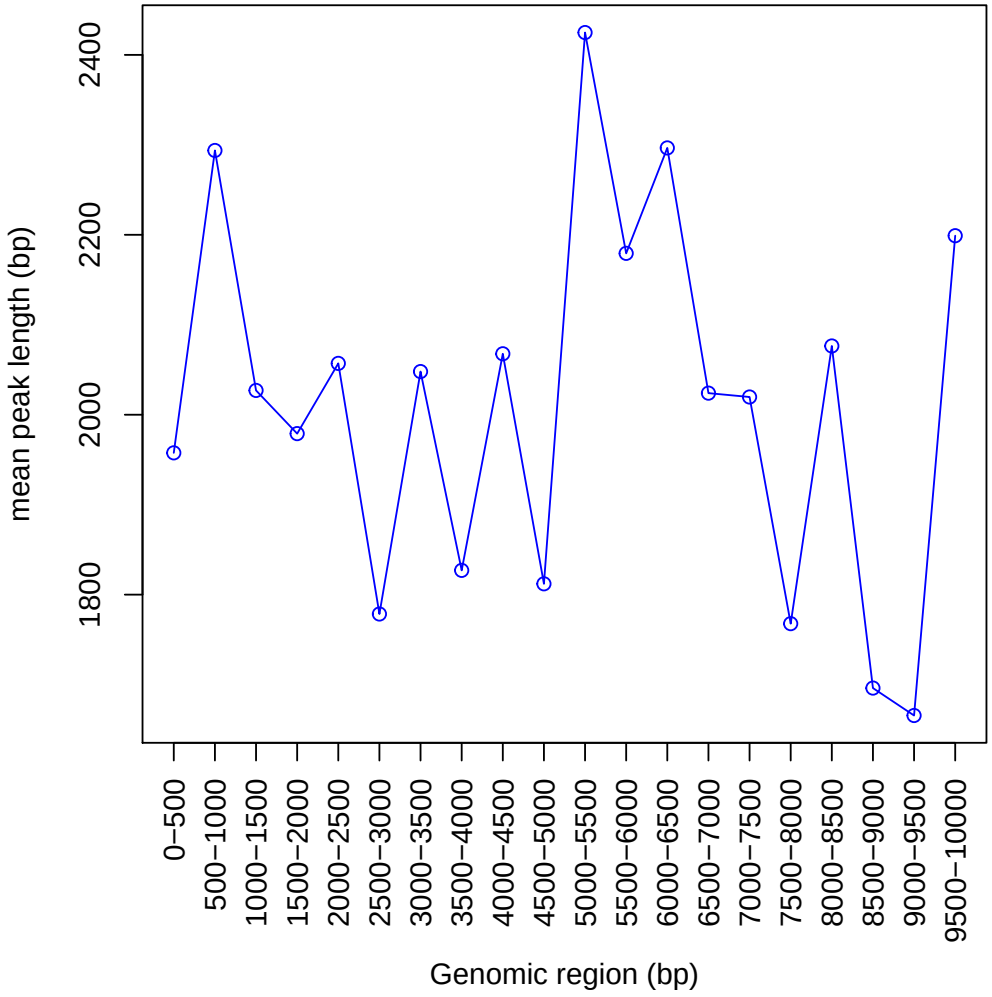



This line plot shows that the concentration of significant peaks in this dataset (Barbier et al. 2016) is highest between 0 and 1000 bp upstream of a gene, with over 90% of peaks in these regions being statistically significant. In contrast, between 1000 bp and 2500 bp, only about half of the total peaks contained in these intervals are significant. Statistical significance then fluctuates noisly at further upstream genomic intervals, but with at least a quarter (25%) of the total peaks in these further upstream regions being statistically significant. As such, the take-home message is that genomic regions within the first 1000 bp upstream of their respective genes are most likely to contain significant peaks (relative to the total peak count in these regions) and are therefore hotspots, but regions beyond this also contain a fair share of statistically significant peaks.

One interesting area to investigate is the variance in the broadness of various significant peaks across different genomic intervals. In other words, asking questions like "are statistically significant peaks that are located very close to their nearest gene (e.g., 0-500 bp away) wider or narrower than peaks located

500-1000 bp away from their nearest gene?". To answer this question we can do:

```
> sigpeaks <- system.file("extdata", "significantpeaksfile.txt", package="geneXtendeR")
> peaksInput(sigpeaks)
> meanPeakLengthPlot(rat, 0, 10000, 500)
```




This line plot displays the mean (average) length of all significant peaks found within each genomic interval. Clearly, the "average peak" is slightly narrower in 0-500 bp intervals than in 500-1000 bp intervals yet, overall, peak lengths tend to fluctuate more or less stochastically at various intervals. To get the exact peak length, we can do:

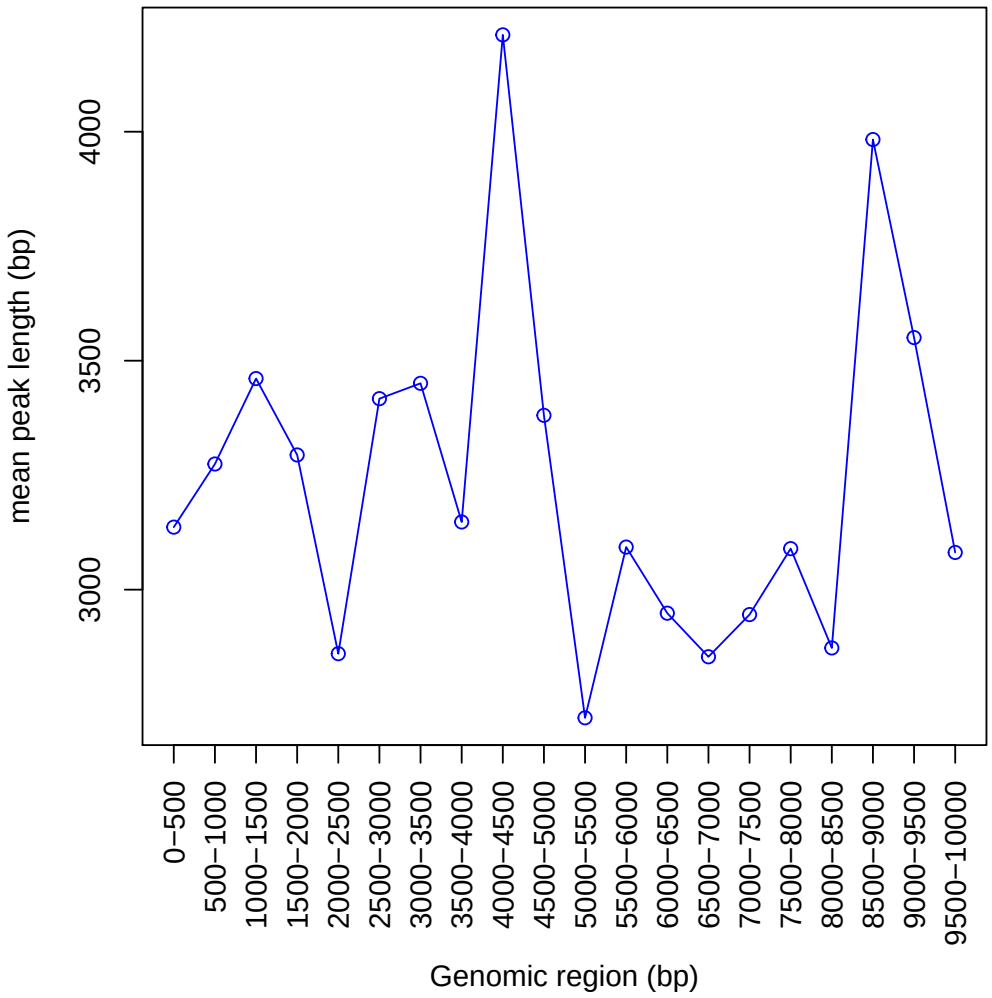
```
> sigpeaks <- system.file("extdata", "significantpeaksfile.txt", package="geneXtendeR")
> peaksInput(sigpeaks)
> meanPeakLength(rat, 0, 500)
```

```
[1] 1957.621
```

So the mean peak length in the interval 0-500 bp is approximately 1958 bp. Although we see that there

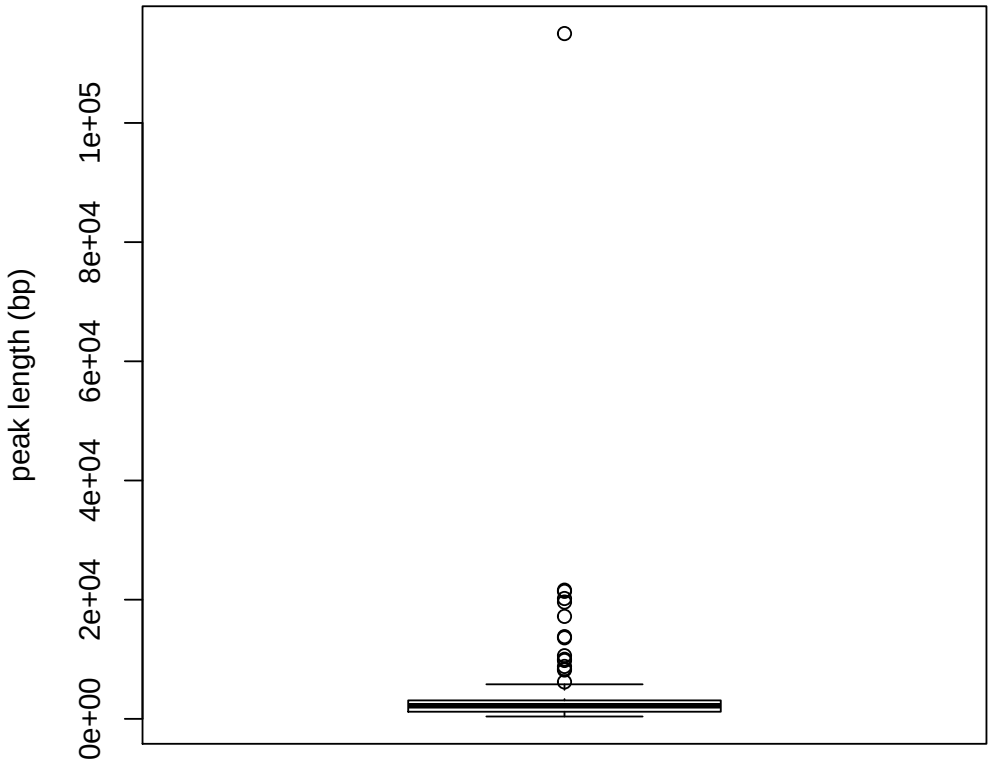
is no specific interval with peaks of extraordinary average lengths, it is still possible to see peak length outliers in certain cases (especially when looking at total peak sets):

```
> allpeaks <- system.file("extdata", "totalpeaksfile.txt", package="geneXtendeR")
> peaksInput(allpeaks)
> meanPeakLengthPlot(rat, 0, 10000, 500)
```




We see that the 4000-4500 bp and 8500-9000 bp intervals both look quite different in terms of their mean peak lengths relative to the other intervals. To see if the mean might be influenced by a strong outlier(s), we can do:

```
> allpeaks <- system.file("extdata", "totalpeaksfile.txt", package="geneXtendeR")
> peaksInput(allpeaks)
> peak_lengths <- peakLengthBoxplot(rat, 4000, 4500)
```




This box-and-whisker plot shows a clear outlier, which is an example of a very broad peak. We can find the exact length of this outlier peak using:

```
> peak_lengths <- peakLengthBoxplot(rat, 4000, 4500)
> max(peak_lengths)
```

```
[1] 114999
```

So this outlier peak measures 114999 bp in total length, therefore making it an extremely broad peak. To see what nearest gene it resides to, we can first extract the peak's index by:

```
> peak_lengths <- peakLengthBoxplot(rat, 4000, 4500)
> match(114999, peak_lengths)
```

```
[1] 126
```

which returns the index of where this peak length is found. Then the following command finds all unique peaks that reside between 4000 and 4500 bp upstream of their nearest gene:

```
> distinct(rat, 4000, 4500)
```

```
      V1        V2        V3  V4        V5        V6                 V7              V8 V9
  1:   1  19526200  19526799   1  19520708  19526671 ENSRNOG00000030796 AABR07000595.1  0
  2:   1  61630800  61631999   1  61624941  61630954 ENSRNOG00000025949        Vom1r22  0
  3:   1  71346800  71347999   1  71334629  71347133 ENSRNOG00000049014   LOC100912263  0
  4:   1  98385400  98394199   1  98394160  98403468 ENSRNOG00000037331           Cd33  0
  5:   1 101099600 101101399   1 101086377 101100094 ENSRNOG00000020583          Fcgrt  0
 ---
124:  18  60006800  60007199  18  59985860  60007069 ENSRNOG00000017852           Nars  0
125:  19  45499400  45499799  19  45499420  45507827 ENSRNOG00000053551 AABR07043877.1  0
126:  19  54877400  54992399  19  54871853  54877469 ENSRNOG00000028578 AABR07044065.1  0
127:  20  30610800  30620799  20  30606026  30611101 ENSRNOG00000049167 AABR07044988.1  0
128: 100  73017400  73018799 100  73018667  73024598 ENSRNOG00000027980 AABR07039245.1  0
```

where we see that index 126 belongs to gene `AABR07044065.1`[3]. Checking the arithmetic difference between column 3 and column 2 for this specific row verifies 114999, as these two columns represent the peak start position and peak end positions. Now let's identify what the other columns represent by running the `distinct()` function again (but this time on a smaller interval to have less output printed to the screen):

```
> fpath <- system.file("extdata", "somepeaksfile.txt", package="geneXtendeR")
> peaksInput(fpath)
> distinct(rat, 2300, 2400)
```

```
    V1        V2        V3 V4        V5        V6                 V7              V8 V9
 1:  1  79718600  79725199  1  79725197  79728613 ENSRNOG00000026891     AC093995.1  0
 2:  1 188715600 188716999  1 188688243 188715680 ENSRNOG00000016013         Gprc5b  0
 3:  1 214368800 214373199  1 214373115 214386385 ENSRNOG00000018367         Taldo1  0
 4:  1 221669800 221671199  1 221671190 221694018 ENSRNOG00000027456        Cdc42bpg  0
 5:  1 236532800 236534799  1 236529431 236532885 ENSRNOG00000022308   LOC103691298  0
 6:  3  82239000  82242199  3  82096568  82239064 ENSRNOG00000008758        Tspan18  0
 7:  3  82780200  82784599  3  82762362  82780214 ENSRNOG00000042533           Accsl  0
 8:  3 146409600 146412399  3 146376328 146409652 ENSRNOG00000006795           Apmap  0
 9:  3 165702800 165706799  3 165678807 165702889 ENSRNOG00000042101           Zfp93  0
10:  4  84850400  84851999  4  84851986  84872257 ENSRNOG00000010205           Mturn  0
11:  4 118157000 118157799  4 118157747 118166562 ENSRNOG00000016273        Fam136a  0
12:  4 171955800 171956999  4 171956961 171961084 ENSRNOG00000057540 AABR07062363.1  0
13:  4 180237200 180239199  4 180231882 180237204 ENSRNOG00000048961         Bhlhe41  0
14:  5  36437600  36438199  5  36433358  36437694 ENSRNOG00000055329 AABR07047528.1  0
15:  5  69038200  69039399  5  69035218  69038218 ENSRNOG00000060997              U6  0
```

[3]This peak may not be statistically significant, but how could it be if it's so huge? In situations like this, it may be a good idea to check what is known about the gene already: http://panthertest2.usc.edu/genes/gene.do?acc=RAT%7CEnsembl=ENSRNOG00000028578%7CUniProtKB=A0A0G2K0W2. Clearly, not much is known yet.

```
16:  5 121456000 121457199  5 121451803 121456072 ENSRNOG00000045614   LOC102552337  0
17:  5 153628200 153630199  5 153568245 153628269 ENSRNOG00000018109          Clic4  0
18:  7  14586000  14587199  7  14587120  14615369 ENSRNOG00000048450        Cyp4f37  0
19:  7  75225000  75225799  7  75225775  75249569 ENSRNOG00000061463 AABR07057510.3  0
20:  8 133130600 133133199  8 133126720 133130690 ENSRNOG00000006730         Ccr1l1  0
21: 10   1830200   1832199 10   1832118   1841132 ENSRNOG00000040121     RGD1565158  0
22: 11  80315400  80316799 11  80316777  80332099 ENSRNOG00000022160           Rtp2  0
23: 14  76654000  76654999 14  76654911  76833661 ENSRNOG00000051169           Clnk  0
24: 14 103716400 103719199 14 103711769 103716440 ENSRNOG00000054704 AABR07016558.1  0
25: 16    631200    642399 16    517332    631224 ENSRNOG00000061982 AABR07024473.2  0
26: 16   9020200   9020999 16   9020987   9055164 ENSRNOG00000042628     RGD1561145  0
27: 16  75363800  75364599 16  75364529  75368406 ENSRNOG00000029462         Defal1  0
28: 20   1747000   1747399 20   1747316   1751142 ENSRNOG00000050043        Olr1735  0
29: 20  22423400  22426199 20  22420251  22423425 ENSRNOG00000057124 AABR07044824.1  0
    V1        V2        V3 V4        V5        V6                 V7             V8 V9
```

This data table shows 29 separate entries sorted by chromosome and start position. `V1-V3` denote the chromosome/start/end positions of the peaks, `V4-V6` denote the respective values for the genes, `V7` is the gene ID (e.g., Ensembl ID), `V8` is the gene name, and `V9` is the distance of each respective peak to its nearest gene. It should be noted that the X chromosome is designated by the integer 100, the Y chromosome by the integer 200, and the mitochondrial chromosome by the integer 300. This is done for sorting purposes (see `?peaksInput` for details). In short, the `distinct()` command finds what peaks-on-top-of-genes would be missed if a 2300 bp upstream extension is used instead of a 2400 bp extension. In other words, these 29 genes all reside between 2300-2400 bp upstream of their nearest gene.

It may be of interest to note the differential gene ontologies between these two upstream extensions:

```
> library(org.Rn.eg.db)
> library(GO.db)
> x <- diffGO(rat, 2300, 2400, BP, org.Rn.eg.db)
> head(x, 20)
```

```
   gene$SYMBOL       GOID                                                    TERM
1       Gprc5b GO:0001934                  positive regulation of protein phosphorylation
2       Gprc5b GO:0007186                    G-protein coupled receptor signaling pathway
3       Gprc5b GO:0007626                                         locomotory behavior
5       Gprc5b GO:0010976            positive regulation of neuron projection development
7       Gprc5b GO:0032147                           activation of protein kinase activity
9       Gprc5b GO:0042593                                         glucose homeostasis
11      Gprc5b GO:0043123 positive regulation of I-kappaB kinase/NF-kappaB signaling
13      Gprc5b GO:0045666                    positive regulation of neuron differentiation
14      Gprc5b GO:0045860                  positive regulation of protein kinase activity
15      Gprc5b GO:0050729                    positive regulation of inflammatory response
17      Gprc5b GO:0060907           positive regulation of macrophage cytokine production
19      Gprc5b GO:0061098         positive regulation of protein tyrosine kinase activity
21      Gprc5b GO:0090263          positive regulation of canonical Wnt signaling pathway
```

```
23      Taldo1 GO:0005975                                carbohydrate metabolic process
24      Taldo1 GO:0006002                     fructose 6-phosphate metabolic process
25      Taldo1 GO:0006098                                  pentose-phosphate shunt
26      Taldo1 GO:0009052               pentose-phosphate shunt, non-oxidative branch
27      Taldo1 GO:0019682                glyceraldehyde-3-phosphate metabolic process
28    Cdc42bpg GO:0006468                                  protein phosphorylation
30    Cdc42bpg GO:0035556                        intracellular signal transduction
```

This dataframe shows the first 20 unique gene ontology terms, their IDs, and respective gene symbols. Clearly, gene name *Gprc5b* has several BP ontologies related explicitly to the brain, while *Taldo1* does not. Considering that the ChIP-seq peaks dataset used as input into `geneXtendeR` comes from a ChIP-seq study investigating the prefrontal cortex, this suggests that a 2400 bp extension may be more suitable for this brain dataset. However, such decisions are left entirely to the discretion and judgment of the user in deciding the relative importance of specific genes and their respective GO terms (BP, CC, or MF) to the goals of the computational analysis (as well as plans for experimental follow-up and validation). See Discussion section for details.

It is also critical to note that the `diffGO()` function returns ALL known gene ontologies, NOT a gene ontology enrichment analysis (more about this in Discussion section). The goal is to provide users with knowledge regarding all possible known roles of any given gene. For example, by knowing that a potential gene candidate has previously been linked with known brain-related ontologies, a user may be prompted to look more closely into the relevant literature behind this gene and its implications to the biological question under study (before embarking on making a decision about its potential impact and suitability as a good candidate for experimental validation).

Furthermore, a user may plot the differential gene ontology results as an interactive network:

```
> library(networkD3)
> library(org.Rn.eg.db)
> library(dplyr)
> makeNetwork(rat, 2300, 2400, BP, org.Rn.eg.db)
```

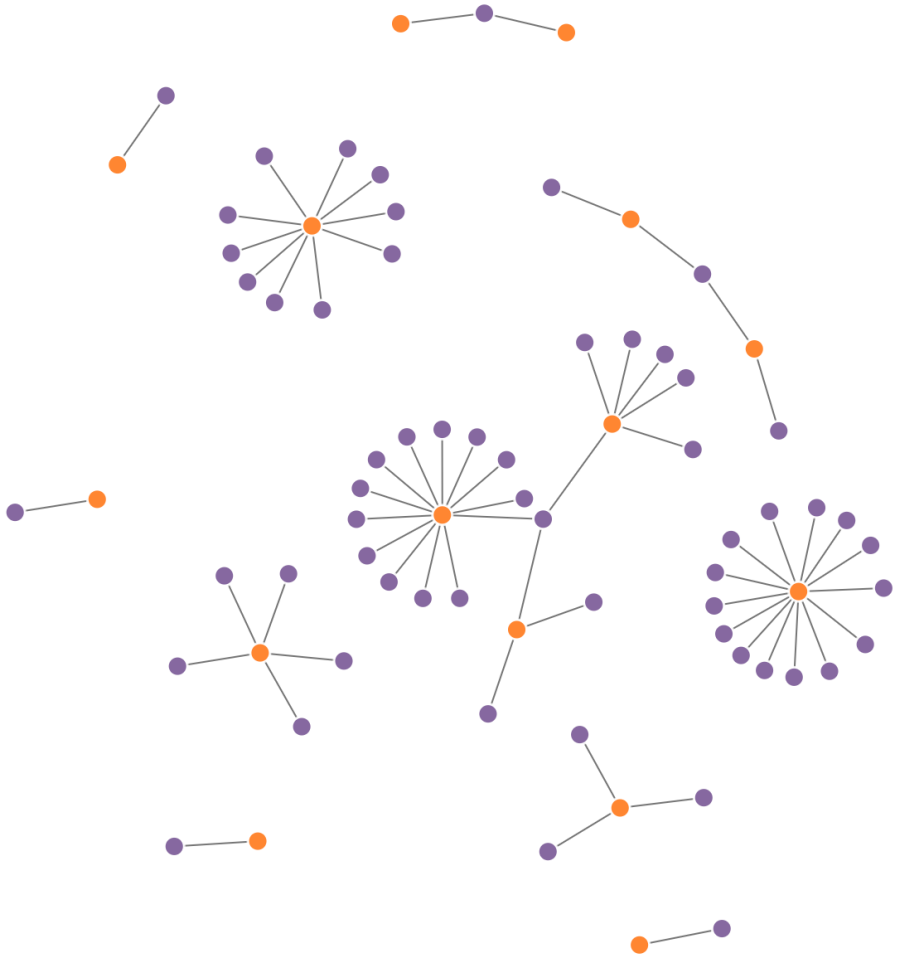


Figure 1: Orange color denotes gene names, purple color denotes GO terms. A user can hover the mouse cursor over any given node to display its respective label directly within R Studio. Likewise, users can dynamically drag and reorganize the spatial orientation of nodes, as well as zoom in and out of them for visual effect.

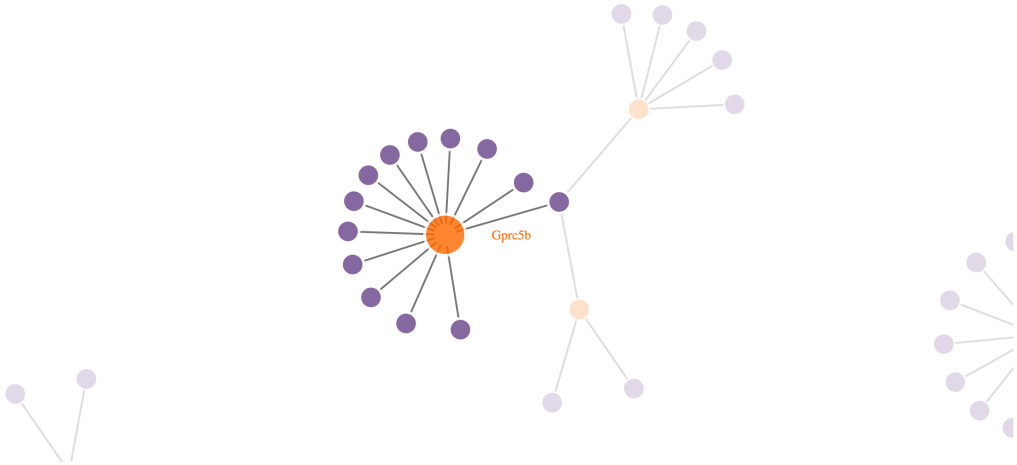



Figure 2: Orange color denotes gene names, purple color denotes GO terms. A user can hover the mouse cursor over any given node to display its respective label directly within R Studio. Likewise, users can dynamically drag and reorganize the spatial orientation of nodes, as well as zoom in and out of them for visual effect.

In addition, users can generate word clouds comprised from words present in their GO terms:

```
> library(tm)
> library(SnowballC)
> library(wordcloud)
> library(RColorBrewer)
> makeWordCloud(rat, 2300, 2400, BP, org.Rn.eg.db)
```


Figure 3: Word cloud generated from words comprising gene ontology terms of category BP. This word cloud shows the words that are used within BP gene ontology terms of peaks found to be present between 2300 and 2400 bp upstream of their nearest genes.

It may also be of interest to visually examine the most frequently used words found within GO terms:

```
> library(tm)
> library(SnowballC)
> library(wordcloud)
> library(RColorBrewer)
> plotWordFreq(rat, 2300, 2400, BP, org.Rn.eg.db, 10)
```

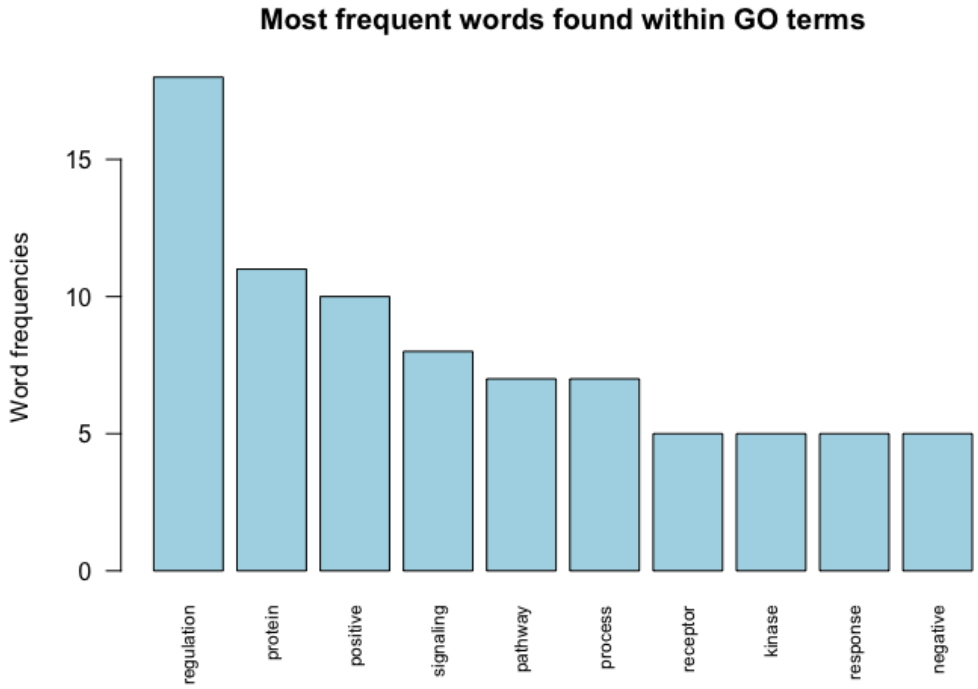



Figure 4: This barplot shows the top 10 words used within gene ontology terms (specific to BP) of peaks found to be present between 2300 and 2400 bp upstream of their nearest genes.

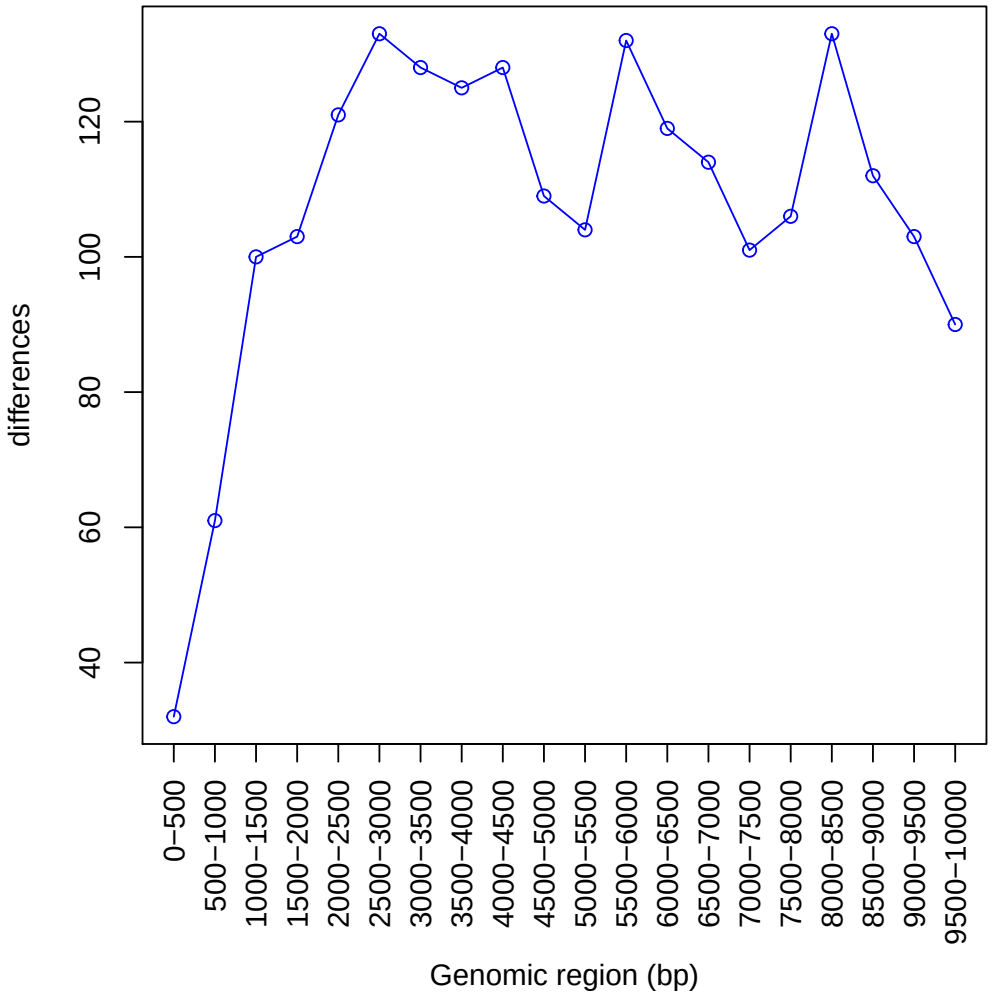
Once the user has chosen the specific upstream extension to be used, the peak file is ready to be fully annotated:

```
> annotate(rat, 2400)
```

which generates a fully annotated peaks outfile (in the user's working directory) containing various genomic features and labeled headers.

## Discussion

Even though `geneXtendeR` is designed to compute (and analyze/display) optimal gene extensions tailored to the characteristics of a specific peak input file, `geneXtendeR` will not explicitly impose on the user the optimal extension to select, since this information is highly study-dependent and, as such, is ultimately reserved to the user's discretion. For example, a user may choose a conservatively lower upstream extension (e.g., for studies investigating narrow peaks such as H3K4me3 or H3K9ac that exhibit a compact and localized enrichment pattern, where high upstream extensions may begin to lose biological relevance). An example of such a user-driven decision would be the selection of a 1500 bp upstream extension instead of a 3500 bp extension in situations like this:




This line plot is derived from the input peak dataset used from the H3K9me1 study examined earlier (Barbier et al. 2016). If the study had examined a narrower chromatin mark (e.g., H3K4me3) then the decision process for choosing an optimal extension may have been different.

In certain cases, additional extensions are unlikely to add significant value to the annotation of the peak file. Taking the example of the 0-10000 bp line plot, an upstream extension beyond 3500 bp globally across every gene in a genome would most likely not accurately reflect the biology of the peak input file (since such large global upstream extensions are likely to reach considerably beyond known proximal promoter elements, especially for relatively narrow histone marks or transcription factors). Such assumptions may be validated directly by the user by investigating the p-value and FDR of specific peaks using a combination of `HT-seq` (to count the reads) and `edgeR/DESeq2` (to assess statistical significance). As such, `geneXtendeR` is designed to be used as part of a biological workflow involving subsequent statistical analysis:

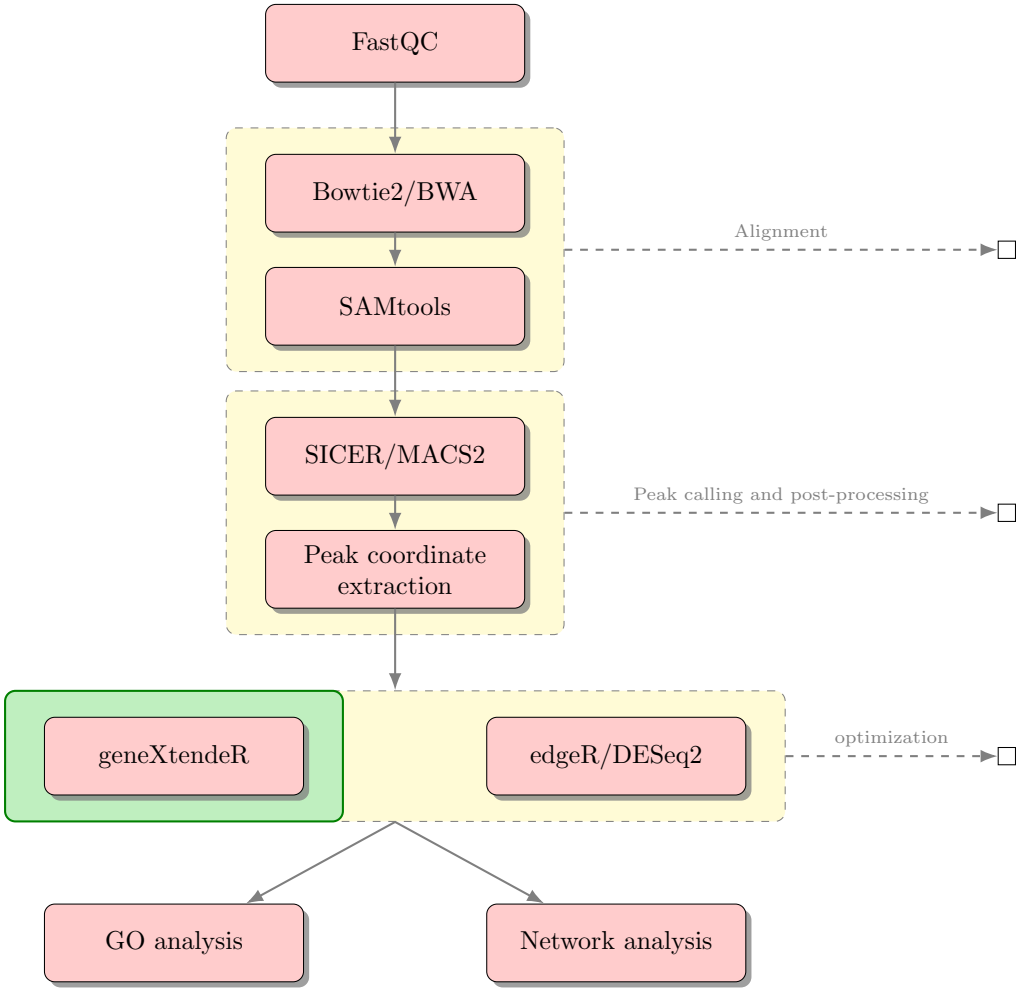
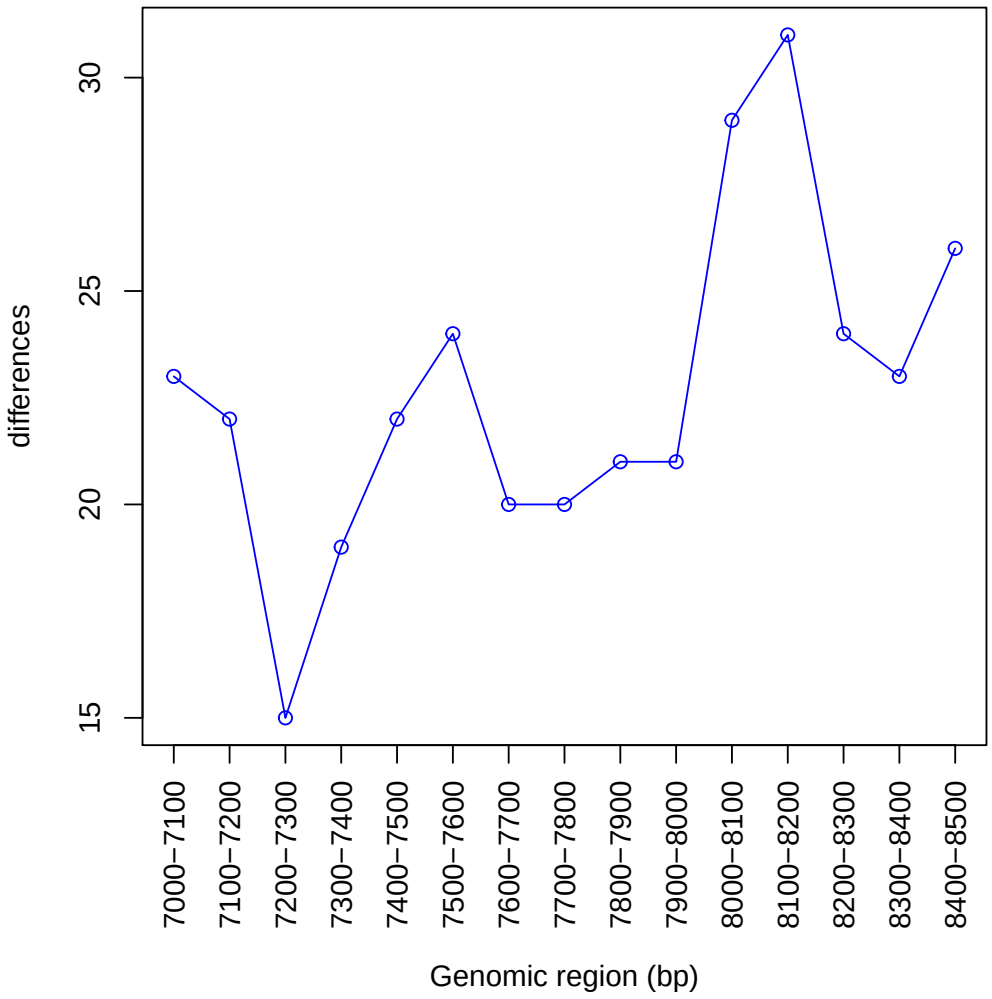



Figure 5: Sample biological workflow using `geneXtendeR` in combination with existing statistical software to analyze peak significance. Subsequent gene ontology enrichment or network analysis may be conducted on genes associated with statistically significant peaks.

It is entirely possible (and probable) for significant peaks to be present at relatively high upstream extension levels (i.e., large gene-spheres), albeit these significant peaks may be associated with biology not directly relevant to the study at-hand, due mainly to the sheer magnitude of the distance of the peak from traditional gene boundaries (where traditional gene boundaries may be loosely defined as $+/-\approx 3$ kb from TSS and $+/-\approx 0.5$kb from TES). Consequently, it is likely for peaks-on-top-of-genes to exhibit higher levels of noise at higher upstream extension levels. Nevertheless, this does not mean that potential enhancer activity should be discounted. For instance, it is not uncommon to see a steady rise or even a surge in the number of peaks-on-top-of-genes at higher upstream extension levels:

```
> linePlot(rat, 7000, 8500, 100)
```




This line plot shows that there are over 30 peaks in this dataset (across the rat genome) that reside between 8100 and 8200 bp upstream of their nearest gene. In far-out cases like this, it is particularly recommended to examine the statistical significance of peaks to get a sense for the possibility of potential enhancer activity/regulation. Of course, such computational findings would require experimental follow-up and/or database mining for known motifs. Assessment of such statistical significance values is beyond the scope of `geneXtendeR`, in order to allow the user freedom to choose the most appropriate statistical package/technique for their analysis. As before, first use the `distinct()` function to create a table of unique genes located under peaks between the two upstream extension levels:

```
> distinct(rat, 8100, 8200)
```

Then, assess the statistical significance of these peaks using a combination of `HT-seq` (Anders et al. 2015) and `edgeR` (Robinson et al. 2010), or `HT-seq` and `DESeq2` (Love et al. 2014), or some other appropriate combination of existing software tools. Genes associated with the resultant statistically significant peaks may then be further assessed with gene ontology enrichment analysis to help answer a variety of interesting research questions. It should once again be noted that the `diffGO()` function

does NOT perform gene ontology enrichment analysis. Instead, it returns all known gene ontologies for each gene. The purpose and utility of this is described in the previous section.

Moreover, DNA sequences under peaks may be checked for the presence of known regulatory motifs (e.g., using TRANSFAC (Matys et al. 2006) or MEME/JASPAR (Sandelin et al. 2004, Bailey et al. 2009)), or for the presence of biological repeats (e.g., using RepeatMasker (Smit et al. 2015)). Pending a prospective GO enrichment and network analysis, functional validation may be followed up in the lab to test any potential regulatory sites or prospective enhancer elements, thereby bringing the computational analysis pipeline back to the bench.

In addition to the computational workflows discussed above, geneXtendeR's wide array of functions makes it possible to conduct some rather interesting and creative combinations of genomic analysis. Let's say, for example, that a user wants to explore all known ontological differences across specific disparate sectors of the genome (e.g., 0-500 bp vs. 2000-3000 bp, but removing 501-1999 bp from consideration). In other words, look at all peaks (across the entire genome) that reside between 0-500 bp upstream of their nearest gene (and 2000-3000 bp upstream of their nearest gene), and extract unique gene ontologies that differ between these two variable-length sectors (where one is 500 bp long and the other is 1000 bp in length). This can be accomplished rather conveniently using dplyr:

```
> library(dplyr)
> library(org.Rn.eg.db)
> library(GO.db)
> a <- diffGO(rat, 0, 500, BP, org.Rn.eg.db)
> b <- diffGO(rat, 2000, 3000, BP, org.Rn.eg.db)
> dplyr::filter(b, TERM %in% a$TERM)
```

| | gene$SYMBOL | GOID | TERM |
|---|---|---|---|
| 1 | Sod2 | GO:0001889 | liver development |
| 2 | Sod2 | GO:0007507 | heart development |
| 3 | Sod2 | GO:0008285 | negative regulation of cell proliferation |
| 4 | Sod2 | GO:0042311 | vasodilation |
| 5 | Sod2 | GO:0042493 | response to drug |
| 6 | Sod2 | GO:0043066 | negative regulation of apoptotic process |
| 7 | Dll1 | GO:0001757 | somite specification |
| 8 | Dll1 | GO:0008284 | positive regulation of cell proliferation |
| 9 | Dll1 | GO:0008285 | negative regulation of cell proliferation |
| 10 | Dll1 | GO:0045596 | negative regulation of cell differentiation |
| 11 | Olr40 | GO:0007186 | G-protein coupled receptor signaling pathway |
| 12 | Hbb | GO:0070527 | platelet aggregation |
| 13 | Olr139 | GO:0007186 | G-protein coupled receptor signaling pathway |
| 14 | Olr282 | GO:0007186 | G-protein coupled receptor signaling pathway |
| 15 | Gprc5b | GO:0007186 | G-protein coupled receptor signaling pathway |
| 16 | Aqp8 | GO:0055085 | transmembrane transport |
| 17 | Aqp8 | GO:0071320 | cellular response to cAMP |
| 18 | Ano9 | GO:1902476 | chloride transmembrane transport |
| 19 | Osbpl5 | GO:0006869 | lipid transport |
| 20 | Cdc42bpg | GO:0006468 | protein phosphorylation |

| | | | |
|---|---|---|---|
| 21 | Dusp5 | GO:0045892 | negative regulation of transcription, DNA-templated |
| 22 | Adgrl2 | GO:0007166 | cell surface receptor signaling pathway |
| 23 | Adgrl2 | GO:0007186 | G-protein coupled receptor signaling pathway |
| 24 | Nfe2l2 | GO:0016567 | protein ubiquitination |
| 25 | Nfe2l2 | GO:0071456 | cellular response to hypoxia |
| 26 | Olr559 | GO:0007186 | G-protein coupled receptor signaling pathway |
| 27 | Tspan18 | GO:0007166 | cell surface receptor signaling pathway |
| 28 | Kcnq2 | GO:0060081 | membrane hyperpolarization |
| 29 | RGD1565415 | GO:0006412 | translation |
| 30 | Reg3b | GO:0008284 | positive regulation of cell proliferation |
| 31 | Reg3b | GO:0043066 | negative regulation of apoptotic process |
| 32 | Olr828 | GO:0007186 | G-protein coupled receptor signaling pathway |
| 33 | Tspan9 | GO:0007166 | cell surface receptor signaling pathway |
| 34 | Bhlhe41 | GO:0045892 | negative regulation of transcription, DNA-templated |
| 35 | Aptx | GO:0006974 | cellular response to DNA damage stimulus |
| 36 | Ccl21 | GO:0007186 | G-protein coupled receptor signaling pathway |
| 37 | Aldob | GO:0001889 | liver development |
| 38 | Aldob | GO:0042493 | response to drug |
| 39 | Clic4 | GO:1902476 | chloride transmembrane transport |
| 40 | Htr1d | GO:0042310 | vasoconstriction |
| 41 | Slc2a5 | GO:0055085 | transmembrane transport |
| 42 | Nlrc4 | GO:0016567 | protein ubiquitination |
| 43 | Esyt1 | GO:0006869 | lipid transport |
| 44 | Sbno2 | GO:0045892 | negative regulation of transcription, DNA-templated |
| 45 | Olr1085 | GO:0007186 | G-protein coupled receptor signaling pathway |
| 46 | Fbxo7 | GO:0016567 | protein ubiquitination |
| 47 | Dnmt1 | GO:0042493 | response to drug |
| 48 | Dnmt1 | GO:0045892 | negative regulation of transcription, DNA-templated |
| 49 | Xcr1 | GO:0007186 | G-protein coupled receptor signaling pathway |
| 50 | Ccr1l1 | GO:0007186 | G-protein coupled receptor signaling pathway |
| 51 | Il17a | GO:0007166 | cell surface receptor signaling pathway |
| 52 | RGD1559955 | GO:0006412 | translation |
| 53 | LOC684471 | GO:0007186 | G-protein coupled receptor signaling pathway |
| 54 | Il3 | GO:0008284 | positive regulation of cell proliferation |
| 55 | Il3 | GO:0043066 | negative regulation of apoptotic process |
| 56 | Olr1501 | GO:0007186 | G-protein coupled receptor signaling pathway |
| 57 | Socs3 | GO:0016567 | protein ubiquitination |
| 58 | Socs3 | GO:0042493 | response to drug |
| 59 | Socs3 | GO:0043066 | negative regulation of apoptotic process |
| 60 | Fbxw8 | GO:0016567 | protein ubiquitination |
| 61 | Fcgr2b | GO:0007166 | cell surface receptor signaling pathway |
| 62 | Eef1e1 | GO:0008285 | negative regulation of cell proliferation |
| 63 | F13a1 | GO:0007596 | blood coagulation |
| 64 | Vom2r66 | GO:0007186 | G-protein coupled receptor signaling pathway |
| 65 | Csnk2a2 | GO:0006468 | protein phosphorylation |

```
66     Csnk2a2 GO:0051726                          regulation of cell cycle
67     Olr1735 GO:0007186       G-protein coupled receptor signaling pathway
```

```
>
```

This displays all biological process (BP) ontologies present in `b` that are not present in `a`. Similarly, one can look at all BP, CC, or MF ontologies present in `a` that are not present in `b`.

## Concluding remarks

`geneXtendeR` is continually evolving, so any suggestions or new feature requests are always appreciated. Likewise, any bug reports may be posted to https://github.com/Bohdan-Khomtchouk/geneXtendeR/issues or emailed to the package maintainer directly.

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