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1 **A computational tool for analysis of mass spectrometry data of ubiquitin enriched samples**

2 Rune Matthiesen^{1*}, Manuel S. Rodriguez² and Ana Sofia Carvalho¹

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4 ¹iNOVA4Health, NOVA Medical School, Faculdade de Ciências Médicas, NMS, FCM,

5 Universidade Nova de Lisboa; Lisboa, Portugal.

6 ²Laboratoire de Chimie de Coordination (LCC)-CNRS, UPS, Toulouse, France.

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8 *) Corresponding author: rune.matthiesen@nms.unl.pt; <https://orcid.org/0000-0002-6353-2616>

9

10 **Running title:** Ubiquitin proteasome analysis in R

11

12 **Key words:** Ubiquitin proteasome system, ubiquitin enrichment, mass spectrometry, R

13 programming language, ubiquitin branch statistics, proximity drugs, proteolysis targeting chimera

14

15

16 **Abstract**

17 Mass spectrometry data on ubiquitin and ubiquitin-like modifiers are becoming increasingly more

18 accessible and the coverage progressively deepen as methodologies mature. This type of mass

19 spectrometry data is linked to specific data analysis pipelines for ubiquitin. This chapter describes a

20 computational tool to facilitate analysis of mass spectrometry data obtained on ubiquitin enriched

21 samples. For example, the analysis of ubiquitin branch site statistics and functional enrichment

22 analysis against ubiquitin proteasome system protein sets are completed with a few functional calls.

23 We foresee that the proposed computational methodology can aid in proximity drug design by, for

24 example, elucidating the expression of E3 ligases and other factors related to the ubiquitin proteasome

25 system.

26

27 **1. Introduction**

28

29 Mass spectrometry (MS) methodologies for studying post translational modifications (PTMs)
30 continue to develop. We might have elucidated the genetic code, but there are a lot of unexplored
31 combinations of PTMs that are poorly understood in terms of their effect on protein function. The
32 number of possible combinations of PTMs on a specific protein grows exponentially with the number
33 of modifications and modification sites we consider in the analysis. For example, given hundreds of
34 possible modifications, each with 5-50 possible modification sites, leads to an astronomically large
35 number of combinations **(1)**. Furthermore, a number of modifications can form heterogeneous branch
36 structures such as for example glycosylation **(2)**, ubiquitination and ubiquitin-like modifiers.
37 Ubiquitination and ubiquitin-like modifiers **(3)** mainly function intracellularly. However, the system
38 is complex and incompletely understood, which constitute one justification for the proposed
39 computational tool presented in this chapter.

40 The design of proximity drugs is based on understanding the ubiquitin proteasome system (UPS),
41 which constitute a vast number of protein families with main players such as ubiquitin and ubiquitin-
42 like modifiers, E1, E2 and E3 ligases, deubiquitinases and the proteasome. For example, the
43 expression level of E3 ligases can vary depending on the cellular state as demonstrated in the analysis
44 in this chapter. Given that proximity drugs are dependent on the presence of a specific targeted ligase,
45 this type of analysis should prove useful in the design process. Furthermore, the majority of the
46 literature on UPS is not systematized. For example, frequently specific proteins are referred to by
47 nonstandard names, making systematic computational analysis complex. One example is FAT10
48 which is often used in the literature but the official gene name is UBD **(4)**. Another example is
49 ubiquitin which is coded by four genes in the human genome, namely RPS27A, UBB, UBC and
50 UBA52. These genes contain one, three, nine and one ubiquitin modifying protein, respectively, all
51 with exactly the same sequence. Therefore, it can be concluded that identification of N terminal di-
52 glycine can either arise from one of the polyubiquitin coding genes or from N-terminal modification

53 with ubiquitin by ubiquitin. The proposed R package contains annotation of the official gene names
54 of protein in the ubiquitin system, which facilitates automated analysis of MS data.

55 Ubiquitin and ubiquitin-like modifiers are covalently bound to amino groups of lysine residues or to
56 the N-terminal of the proteins that they modify. Ubiquitin and ubiquitin-like modifiers also contain
57 lysine residues that, in turn, can be modified. Specific branch linkages of ubiquitin have been linked
58 to specific molecular functions. Although, the system is far more complex, having heterogeneous
59 linkage structures that mix ubiquitin with ubiquitin-like modifiers. Furthermore, ubiquitin can also
60 undergo other modifications such as acetylation and phosphorylation (3). The proposed R package
61 contains functions that automatically estimate the overall average level of linkages in each sample
62 for cross comparisons. This tool was developed based on our past research on tandem ubiquitin
63 binding entities (TUBEs) combined with MS (5-7). However, it can be applied to basically all the
64 current ubiquitin enrichment strategies that currently are being combined with MS identification (*see*
65 **Figure 1**). These technologies TUBEs (8), UbiSite (9), His₆-tag enrichment (10) and *in vivo*
66 biotinylation of ubiquitin (11). Each of these technologies can be combined with additional di-glycine
67 enrichment. The R package “ubiquitin proteasome system in R based on mass spectrometry”
68 (UPSRM) is demonstrated here with a data set obtained using a recent technology called UBISITE
69 for large scale enrichment of ubiquitin modified peptides combined with MS identification (12).
70 However, UPSRM works for all enrichment strategies that end with identification of di-glycine by
71 MS.

72 The output results of R package UPSRM compare ubiquitin occupancy branch sites on ubiquitin and
73 ubiquitin like modifiers in different conditions. For example, cell treatment with protease inhibitors.
74 Overall, these comparisons can potentially highlight the main ubiquitin branches affected by a
75 specific drug targeting ubiquitin ligases, deubiquitinases or more recently the proteolysis targeting
76 chimera (PROTAC) drugs targeting protein degradation using the ubiquitin code.

77

78

79 **2. Materials**

80

81 1. R Cran (<https://cran.r-project.org/>)

82 2. R studio (optional - <https://www.rstudio.com/>)

83 3. R packages: ggpubr, plyr, gplots, ComplexHeatmap.

84 4. MaxQuant (**13**) data: “GlyGly (K)Sites.txt” and “proteinGroups.txt” (*see Note 1*). For this tutorial

85 the data set PXD027328 in ProteomeXchange was used. Only the two text files “GlyGly (K)Sites.txt”

86 and “proteinGroups.txt” are necessary to download.

87

88 **3. Methods**

89

90 **3.1 Software setup and installation of R packages**

91 1. Follow the instructions in the provided links to install R Cran.

92 2. Run the below commands in an R session to install UPSRM:

93 `install.packages("ggpubr")`

94 `install.packages("plyr")`

95 `install.packages("gplots")`

96 `install.packages("ComplexHeatmap")`

97 `install.packages("remotes")`

98 `library(remotes)`

99 `remotes::install_github("ruma1974/UPSRM@main")`

100 3. Load all packages needed (*see Note 2*):

101 `library(UPSRM)`

102 `library(ComplexHeatmap)`

103

104 **3.2 Perform UPS protein expression analysis**

105 The example analysis is performed on a recent data set in PXD027328. The data set consists of U2OS
106 cell lines treated with DMSO, MG132 (proteasome inhibitor), PR619 (DUB inhibitor) or TAK243
107 (UAE inhibitor). The below steps demonstrate the R commands to run and the expected output.

108

109 1. Provide the directory with the MaxQuant text output files

110 `Dir="[Directory]"`

111 2. The command below automatically loads the two data files into R.

112 `res=loadMaxQuant(Dir)`

113 3. Run the command below to see the protein groups available.

114 `showUPSgroups()`

115 Output (*see Note 3*):

116 "Proteasome", "SUMO", "DUBs", "immunoproteasome", "ProteasomeRegulators"

117 "ProteasomeActivators", "Ub", "E1", "E1sumo", "E2", "E3", "Ubl"

118 4. Annotated genes in a specific UPS group can be retrieved with for example:

119 `showGenes("E3")`

120 5. Quantitative LFQ values for the E3 ligases can be extracted with:

121 `M=getQdata(res,"E3")`

122

123 ***3.3 Visualize quantitative UPS data in R***

124 The R package “ComplexHeatmap” provides useful functions for visualizing numeric matrix data.

125

126 1. Rename columns to remove redundant information.

127 `colnames(M)=c("DMSO1", "DMSO2", "DMSO3", "MG1", "MG2", "MG3", "PR1", "PR2", "PR3",`

128 `"TAK1", "TAK2", "TAK3")`

129 2. Define sample conditions

```
130 Fac=c("DMSO", "DMSO", "DMSO", "MG", "MG", "MG", "PR", "PR", "PR", "TAK", "TAK",
131 "TAK")
```

132 3. Use sample condition information to generate a heatmap annotation.

```
133 ha = ComplexHeatmap::HeatmapAnnotation(Sample = Fac,col = list(Sample = c(DMSO = "red",
134 MG = "green",PR="brown", TAK = "blue")),na_col = "black")
```

135 4. Plot the expression heatmap of E3 ligases to generate **Figure 2**.

```
136 x11(w=20,h=10) # only need on linux systems
```

```
137 ComplexHeatmap::Heatmap(log2(M+1), col=gplots::redgreen(255), name = "LFQ", top_annotation
138 =ha,show_row_names =FALSE)
```

139

140 We observe that E3 ligase levels are heavily affected by treatment conditions in the same cell line.

141 This illustrates the need to profile E3 ligases in specific cell conditions for proximity drug design.

142

143 ***3.4 Comparative average branch topology for ubiquitin***

144 The below code demonstrates how UPSRM convince functions can be used to explore average
145 ubiquitin branch topology across sample conditions. It assumes that the MaxQuant data is already
146 loaded (see section 3.2).

147

148 1. The possible ubiquitin or ubiquitin like modifiers that can be analyzed can be retrieved by:

```
149 UPSRM::showUBIs()
```

150 2. Next we define the comparisons to perform (DMSO is used as reference in below example):

```
151 comp <- list( c("MG", "DMSO"), c("PR", "DMSO"), c("TAK", "DMSO") )
```

```
152 Fac=c("DMSO", "DMSO", "DMSO", "MG", "MG", "MG", "PR", "PR", "PR", "TAK", "TAK",
153 "TAK")
```

154 3. Boxplot summarizing average ubiquitin branch sites across conditions is written to a pdf file by
155 the below command (see **Figure 3**):

156 `M=getUbQdata(res,"Ub",Fac,comp,"Q_Ubsites.pdf")`

157 4. Boxplot summarizing average SUMO1-ubiquitin branch sites across conditions is written to a pdf
158 file by the command below (*see Figure 4*):

159 `M=getUbQdata(res,"Ub",Fac,comp,"Q_SUMO1sites.pdf")`

160

161 **4. Notes**

162

163 1. The file “GlyGly (K)Sites.txt” is optional. If only “proteinGroups.txt” is provided then expression
164 analysis of UPS factors is still possible. The columns in “GlyGly (K)Sites.txt” must include
165 “Intensity.[sample]”, “Gene.names”, “GlyGly..K..Probabilities”. The columns in “proteinGroups.txt”
166 must contain “LFQ.[sample]” and “Gene.names”. If interest exists the authors will update the R
167 package with support for other database dependent search engines.

168 2. The other installed R packages are used by UPSRM and will be loaded indirectly when using
169 UPSRM functions.

170 3. The genes annotated to each UPS group will likely change overtime as more knowledge is
171 generated on UPS. We consider the current annotations as core annotations but we will be pleased to
172 receive user feedback to update UPSRM with additional UPS groups.

173

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184

185

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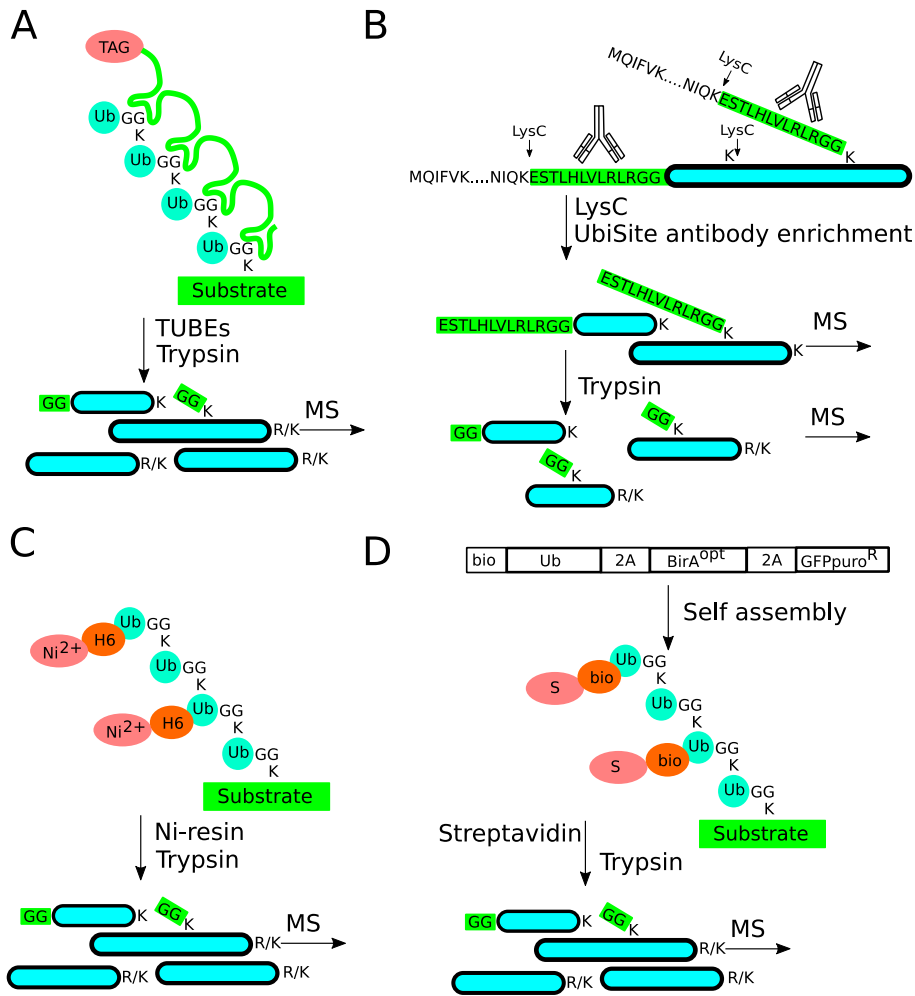
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223

224

225

226 **Figures**



228 **Figure 1. Different enrichment strategies of ubiquitinated proteins and peptides.** A) Strategy
 229 based on tandem ubiquitin binding entities, B) UbiSite, C) Ni²⁺ His6-tag affinity enrichment and D)
 230 *in vivo* biotinylation-Ub followed by streptavidin enrichment.

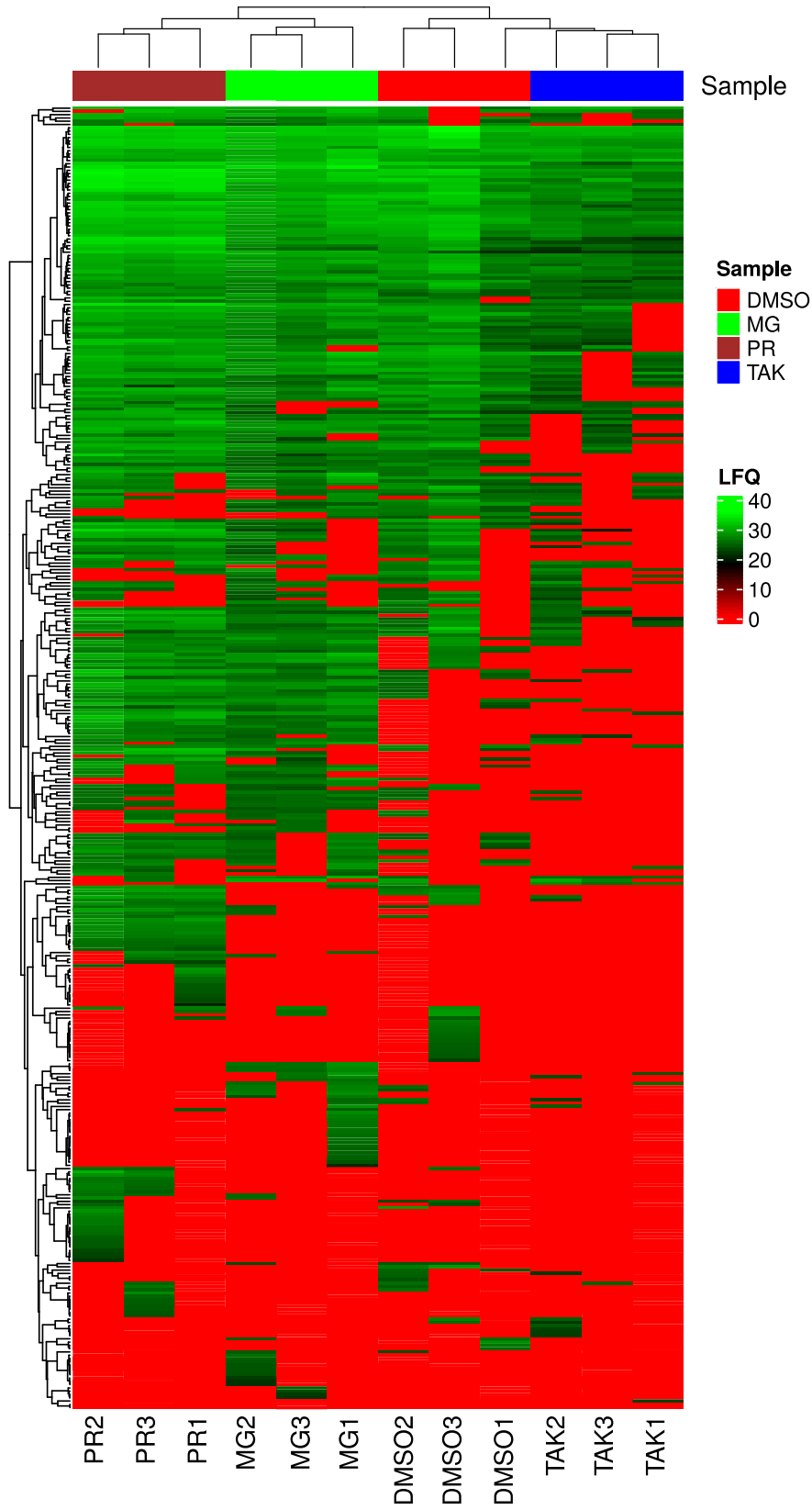


Figure 2. Expression of E3 ligases across sample treatment groups.

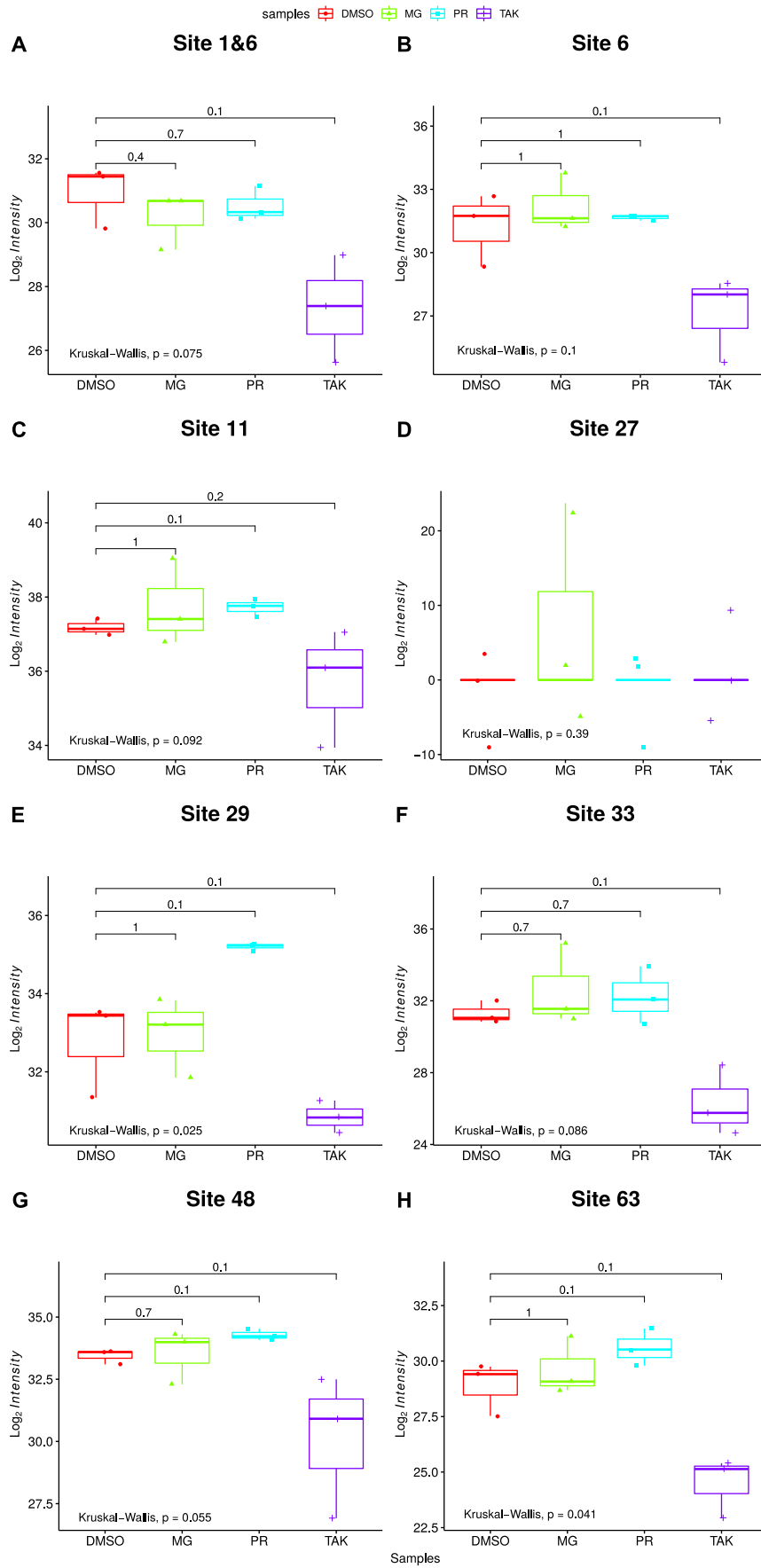


Figure 3. Average ubiquitin branch sites across sample conditions.

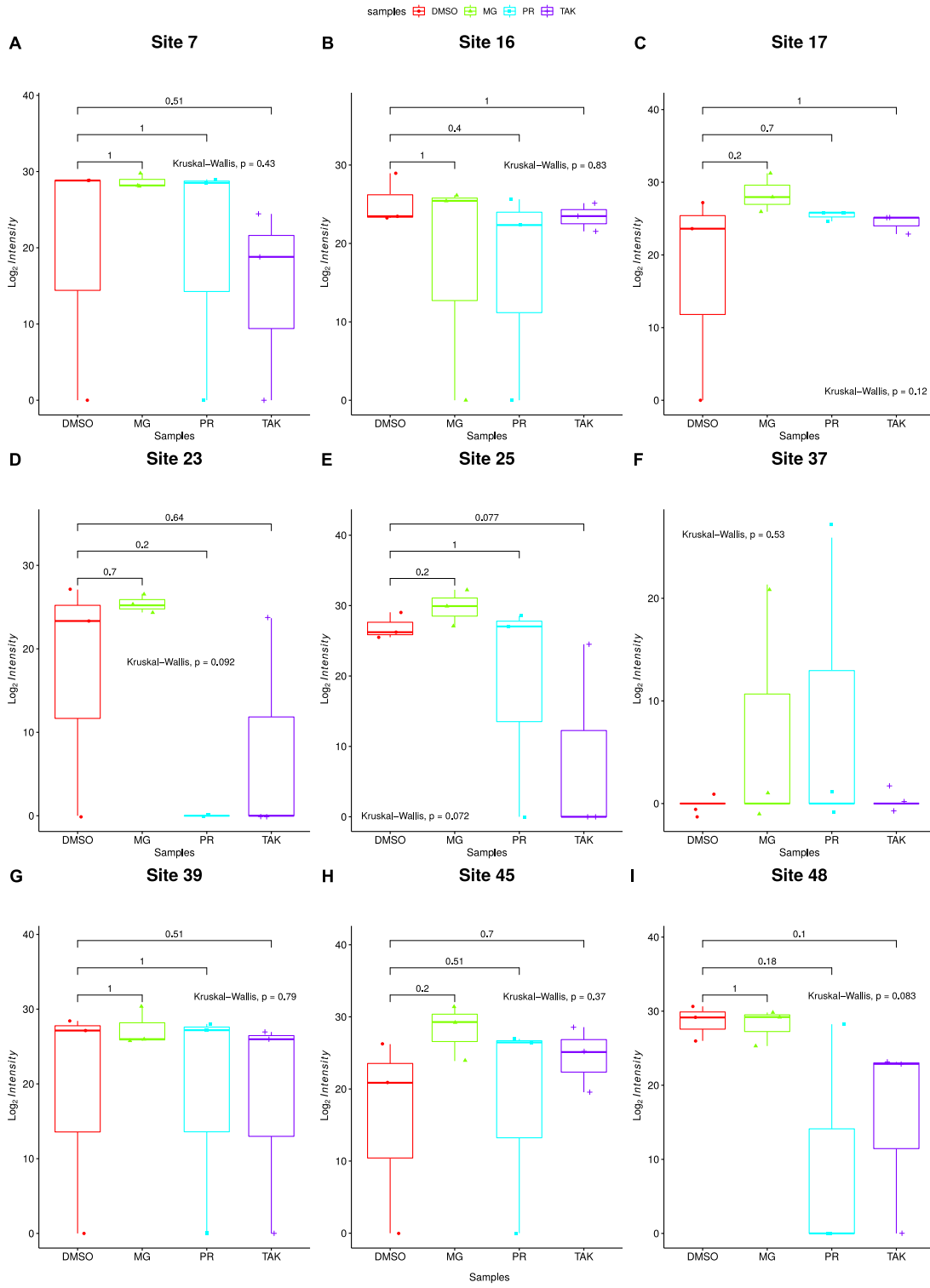


Figure 4. Average ubiquitin-SUMO1 branch sites across sample conditions.