

1 **A non-canonical RNAi pathway controls virulence and** 2 **genome stability in Mucorales**

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7 **Abstract**

8 Epimutations in fungal pathogens are emerging as novel phenomena that could
9 explain the fast-developing resistance to antifungal drugs and other stresses. These
10 epimutations are generated by RNA interference (RNAi) mechanisms that transiently
11 silence specific genes to overcome stressful stimuli. The early-diverging fungus *Mucor*
12 *circinelloides* exercises a fine control over two interacting RNAi pathways to produce
13 epimutants: the canonical RNAi pathway and a new RNAi degradative pathway. The
14 latter is considered a non-canonical RNAi pathway (NCRIP) because it relies on RNA-
15 dependent RNA polymerases (RdRPs) and a novel ribonuclease III-like named R3B2 to
16 degrade target transcripts. Here in this work, we uncovered the role of NCRIP in
17 regulating virulence processes and transposon movements through key components of the
18 pathway, RdRP1, and R3B2. Mutants in these genes are unable to launch a proper
19 virulence response to macrophage phagocytosis, resulting in a decreased virulence
20 potential. The transcriptomic profile of *rdrp1Δ* and *r3b2Δ* mutants revealed a pre-
21 exposure adaptation to the stressful phagosomal environment even when the strains are
22 not confronted by macrophages. These results suggest that NCRIP represses key targets
23 during regular growth and release its control when the fungus is challenged by a stressful
24 environment. NCRIP interacts with the RNAi canonical core to protect genome stability
25 by controlling the expression of centromeric retrotransposable elements. In the absence

26 of NCRIP, these retrotransposons are robustly repressed by the canonical RNAi
27 machinery; thus, supporting the antagonistic role of NCRIP in containing the
28 epimutational pathway. Both interacting RNAi pathways might be essential to govern
29 host-pathogen interactions through transient adaptations, contributing to the unique traits
30 of the emerging infection mucormycosis.

31 **Author summary**

32 Mucormycosis is an emergent and lethal infectious disease caused by Mucorales, a fungal
33 group resistant to most antifungal drugs. *Mucor circinelloides*, a genetic model to
34 characterize this infection, can develop drug resistance via RNAi epimutations. This
35 epimutational RNAi mechanism interacts with a novel non-canonical RNAi pathway
36 (NCRIP), where the ribonuclease III-like R3B2 and the RNA-dependent RNA
37 polymerase RdRP1 are essential. The analysis of the transcriptomic response to
38 phagocytosis by macrophage in *rdp1* Δ and *r3b2* Δ mutants revealed that NCRIP might
39 control virulence in *M. circinelloides*. These mutants showed constitutive activation of
40 the response to phagocytosis and a reduction in virulence in a mouse model, probably
41 caused by a disorganized execution of the genetic program to overcome host defense
42 mechanisms. The antagonistic role of the NCRIP and the RNAi canonical core is evident
43 during post-transcriptional regulation of centromeric retrotransposons. These
44 retrotransposons are silenced by the canonical RNAi pathway, but this regulation is
45 restrained by NCRIP, proven by an overproduction of small RNAs targeting these loci in
46 NCRIP mutants. These new insights into the initial phase of mucormycosis and
47 transposable element regulation point to NCRIP as a crucial genetic regulator of
48 pathogenesis-related molecular processes that could serve as pharmacological targets.

49 **Introduction**

50 Mucorales are a group of ancient fungi that are emerging as a new source of
51 pathogens causing the fungal infection mucormycosis. This infectious disease is
52 increasing the focus of recent studies due to its high mortality rates, which can reach up
53 to 90% in cases of disseminated infection [1,2]. The elevated mortality rate is a direct
54 connection to a lack of effective antifungal treatments, a consequence of the unusual
55 resistance observed in these fungi. In this regard, a novel RNAi-dependent epimutational
56 mechanism of drug resistance has been described in *M. circinelloides* [3]. In this
57 mechanism, *M. circinelloides* generates strains resistant to the antifungal drug FK506
58 after only four days of exposure. The mechanism behind this rapid adaptation relies on
59 the specific silencing of the *fkbA* gene and its encoded FKBP12 protein, which is the
60 target of FK506. Thus, in the absence of FKBP12 due to *fkbA* silencing, the drug FK506
61 is unable to hinder the mycelial growth of *M. circinelloides*, generating transient resistant
62 strains that arise due to selective pressure. The epimutational drug resistance in *M.*
63 *circinelloides* is becoming clinically relevant because epimutants can emerge upon
64 exposure to other antifungal drugs [4], and they exhibit organ-specific stability during in
65 vivo infection [5].

66 The RNAi pathway involved in this epimutation-based drug resistance depends
67 on the canonical components of the RNAi machinery, which are broadly characterized in
68 *M. circinelloides* [6]. First, RNA dependent RNA polymerases (RdRPs) generate double-
69 stranded RNA (dsRNA). Later, dsRNA is processed by RNase III Dicer enzymes to
70 generate small RNAs (sRNAs). Then, the third element of the RNAi canonical core, the
71 Argonaute protein (Ago), uses the sRNAs to conduct homology-dependent repression of
72 the target sequences [7]. Besides drug-resistance, the canonical core elements participate
73 in RNAi-based defensive pathways protecting genomic integrity against invasive nucleic

74 acids and transposable elements, as well as in other RNAi pathways involved in the
75 endogenous regulation of target mRNAs [8].

76 Although epimutants can arise in wild-type strains, the phenomenon is enhanced
77 by mutations in key genes of an RdRP-dependent Dicer-independent degradation
78 mechanism for endogenous mRNA [3,9]. This could mean that either this novel RNAi
79 pathway directly represses the epimutation machinery or that it competes for the same
80 target mRNAs. This degradation mechanism is considered a non-canonical RNA
81 interference pathway (called NCRIP) because it does not share the canonical core RNAi
82 machinery. Indeed, mutational analyses showed that only RdRP enzymes, but neither
83 Dicer nor Argonaute, participate in the NCRIP pathway [10]. The cleaving activity
84 required to degrade target mRNAs relies on a new RNase III-like protein named R3B2,
85 which plays the primary RNase role in the NCRIP pathway. The unique role of RdRPs
86 (RdRP1, RdRP2, and RdRP3) in RNA degradation suggests that the NCRIP mechanism
87 represents a first evolutionary link connecting mRNA degradation and post-
88 transcriptional gene silencing [9].

89 The role of NCRIP in regulating the RNAi-dependent epimutational mechanism
90 emphasizes the intricate network of interactions among RNAi pathways in fungi.
91 However, the actual functional role of NCRIP in cellular processes and the importance of
92 its regulatory effects on fungal physiology are still unknown. The large number of
93 predicted genes that might be regulated by NCRIP suggested a pleiotropic role in fungal
94 physiology, controlling several and diverse processes. Indeed, phenotypic analysis of
95 mutants lacking the NCRIP pathway revealed two prominent phenotypes associated with
96 the lack of NCRIP: *in vitro* oxidative stress resistance and reduced production of
97 zygospores during sexual development [10].

98 RNAi-related mechanisms are important for the maintenance of genome stability
99 and transposon movement in other fungal pathogens such as *Cryptococcus neoformans*
100 [11]. In this basidiomycete, the canonical RNAi machinery plays a protective role by
101 silencing transposable elements during mating, ensuring the genomic integrity of the
102 progeny. A recent study in *M. circinelloides* also found an essential role for the canonical
103 RNAi core in silencing repetitive pericentric transposable elements [12]. Interestingly,
104 analysis of genome-wide sRNA content in epimutants that were deficient in NCRIP
105 revealed an alteration of sRNA levels derived from transposable elements [4]. These
106 studies reinforce the hypothesis of an inhibitory function of NCRIP over the canonical
107 pathway during the production of epimutants. Thus, NCRIP could have a role in
108 maintaining genome integrity through its competitive regulation of the canonical RNAi
109 in the control of transposable elements. Moreover, the resistance to oxidative stress
110 observed *in vitro* in NCRIP deficient mutants [10] could play a specific role for survival
111 in stressful environments, such as those related to the host-pathogen interaction. NCRIP
112 may also be involved in pathogenesis given the high frequency of drug-resistant
113 epimutants in mutants of this pathway, suggesting that this regulatory mechanism could
114 be linked to virulence in *M. circinelloides*.

115 Here, we show a detailed functional analysis of the NCRIP pathway, addressing
116 the functional roles that it might play in fungal biology and pathogenesis. Consequently,
117 we studied the complex network of genes regulated by NCRIP during saprophytic growth
118 and macrophage phagocytosis. This study identifies the complete profile of genes and
119 functional categories regulated by NCRIP in both conditions. Interestingly, most of the
120 fungal genes regulated by phagocytosis are under control of NCRIP, indicating that this
121 RNAi-based mechanism is a master regulator of the response of the pathogen to
122 phagocytosis.

123 **Results**

124 **NCRIP preferentially regulates functional processes during non-stressful conditions**

125 The higher resistance to oxidative stress of *M. circinelloides* NCRIP-deficient
126 mutants prompted us to identify the genes controlled by this RNAi pathway in response
127 to the oxidative burst of macrophages during phagocytosis. To this end, we performed a
128 transcriptomic analysis of the gene expression profiles obtained from high-throughput
129 sequencing of mRNA (RNA-seq) from spores of the wild-type strain and mutants lacking
130 NCRIP activity (*r3b2Δ* or *rdrp1Δ*). The spores were single-cultured in rich medium L15
131 (saprophytic conditions), and co-cultured with the J774A.1 cell-line of mouse
132 macrophages (1.5:1 spore–macrophage ratio) for 5 hours to ensure that most of the spores
133 were phagocytosed. These macrophage samples represent the closest in vitro environment
134 to a clinical in vivo context in which the germinating spores must rapidly overcome
135 oxidative stress to escape from the innate immune response.

136 Messenger RNA was isolated and deep sequenced to analyze the transcriptional
137 response of the control wild-type samples with or without macrophages (Fig 1A, WTM
138 or WTC, respectively), and the two mutant samples, with macrophages (Fig 1A, *r3b2ΔM*
139 and *rdrp1ΔM*) or without (Fig 1A, *r3b2ΔC* and *rdrp1ΔC*). We performed a principal
140 component analysis of the expression values for all genes (mean CPM > 1.0 per gene in
141 all conditions) to further study the variability among the samples (Fig 1A). This analysis
142 revealed that the NCRIP mutant strains, *r3b2Δ* and *rdrp1Δ*, clustered closely together and
143 had a distinct transcriptomic profile compared to the wild-type strain growing in
144 saprophytic conditions without macrophages. However, when the macrophages
145 phagocytosed the spores, the mRNA repertoire of all strains formed a closer cluster and
146 showed a more similar profile. To identify these changes in gene expression, the genetic
147 profiles of the two mutants were compared to the wild-type strain in the presence or

148 absence of macrophages (S1 Dataset). A threshold of a corrected p-value under 0.05
 149 (False Discovery Rate [FDR] of 0.05) and a \log_2 FC $\geq |1.0|$ was selected to consider
 150 differentially expressed genes (DEGs). The deletion of either the *r3b2* or the *rdrp1* gene
 151 caused a profound variation in the mRNA profiles of *M. circinelloides*, especially when
 152 the fungus grows without macrophages (Table 1). Under these saprophytic conditions,
 153 most DEGs trend towards upregulation in the wild-type strain, as expected from the direct
 154 repressive activity of NCRIP. However, downregulation modestly prevailed in the wild-
 155 type spores phagocytosed by macrophages, suggesting repression of a few primary direct
 156 targets of NCRIP that control a vast network of secondary targets (Table 1).

157 **Table 1. Differentially expressed genes in NCRIP mutant strains compared with**
 158 **the wild-type strain**

| Culture conditions | Strain | Upregulated genes ¹ | | Downregulated genes ² | |
|----------------------|----------------|--------------------------------|----------------------------------|----------------------------------|----------------------------------|
| | | # | Average \log_2 FC ³ | # | Average \log_2 FC ³ |
| L15 5h 37 °C | <i>r3b2</i> Δ | 1685 | 2.42 ± 1.36 | 850 | -1.55 ± 0.79 |
| | <i>rdrp1</i> Δ | 1905 | 2.46 ± 1.43 | 1452 | -1.67 ± 0.79 |
| L15 5h 37 °C + Mφ | <i>r3b2</i> Δ | 80 | 1.77 ± 1.49 | 92 | -1.64 ± 0.76 |
| | <i>rdrp1</i> Δ | 77 | 1.86 ± 1.51 | 106 | -1.61 ± 0.84 |

159 ¹FDR \leq 0.05, \log_2 FC \geq 1.0, average \log_2 CPM \geq 1.0
 160 ²FDR \leq 0.05, \log_2 FC \geq -1.0, average \log_2 CPM \geq 1.0
 161 ³Average of all \log_2 fold change values and standard deviation

162 Subsequently, we searched for shared DEGs in both NCRIP mutants compared to
 163 their wild-type control, representing all comparisons in a four-way Venn diagram (Fig
 164 1B). The analysis revealed a total of 2199 genes ($>$ 18% of the genome) regulated by both
 165 R3B2 and RdRP1 under all conditions (Fig 1B, 28 + 16 + 13 + 0 + 49 + 39 + 1 + 33 +
 166 2020), representing 69% of all of the DEGs. These results suggest that NCRIP regulates
 167 these genes, whereas the genes misregulated in only one or the other mutant could be the
 168 result of other independent functions of R3B2 and RdRP1. In saprophytic conditions, the
 169 two mutants showed 2141 DEGs (Fig 1B, 2020 + 39 + 49 + 33), whereas the differences
 170 were more subtle in the phagocytosed spores, and only 93 shared DEGs were identified
 171 (Fig 1B, 28 + 16 + 49). A total of 49 genes were differentially expressed in both *r3b2*Δ

172 and *rdrp1* Δ mutants regardless of the presence or absence of macrophages, and these
173 genes might be regulated by NCRIP and involved in essential processes required under
174 all conditions (Fig 1B). These higher differences without macrophages and the low
175 number of DEGs in the presence of macrophages are in accord with the results observed
176 in the principal component analysis (Fig 1A).

177 To survey the possible cellular processes controlled by the NCRIP machinery in
178 saprophytic growth without any challenge, an enrichment analysis of Eukaryotic
179 Orthologous Groups (KOG) terms was conducted. Under these non-stressful conditions,
180 we found an enrichment in processes related to the production of extracellular structures
181 and secondary metabolites, the remodeling of energy, amino acids, lipids and
182 carbohydrates metabolic pathways, and by contrast an overall reduction in cytoskeletal
183 processes (Fig 2). The genes grouped in these KOG classes indicated specific functional
184 roles not required during phagocytosis and controlled by NCRIP. There was not any
185 shared enrichment in any KOG class after phagocytosis of the two mutants' spores,
186 possibly because the gene set was too small to produce a significant result. Instead, *r3b2* Δ
187 and *rdrp1* Δ mutants showed independent roles in amino acid transport and metabolism,
188 and chromatin structure and dynamics, respectively, suggesting that these genes perform
189 specific roles required during phagocytosis that are not controlled by NCRIP. Altogether,
190 these results indicated a preferential activity of NCRIP under non-stressful conditions,
191 when the spores are cultured without macrophages.

192 **NCRIP repressed the genetic response to phagocytosis during non-stress conditions**

193 Previous studies revealed an intricate network of genes activated in response to
194 phagocytosis, which is essential for the pathogenic potential of Mucorales [13].
195 Considering the large number of genes regulated by NCRIP, and the functional processes

196 involved, we postulated that some of these genes might participate in the response to
197 phagocytosis. To address this hypothesis, we analyzed the DEGs detected in response to
198 phagocytosis in the wild-type strain and the *rdp1* Δ and *r3b2* Δ mutants and presented the
199 results in a three-way Venn's diagram (Fig 3A). The most marked result from this analysis
200 is the high number of genes (a total of 908 out of 1156) differentially expressed only in
201 the wild type during phagocytosis, but not in the *rdp1* Δ or *r3b2* Δ mutant. Therefore,
202 these results identified a broad set of genes responding to phagocytosis in the wild-type
203 strain that is unable to respond in the mutants lacking the NCRIP pathway. Two
204 alternative possibilities could explain these results: either these genes required a
205 functional NCRIP for their activation during phagocytosis or NCRIP is repressing them
206 under non-stressful conditions without macrophages.

207 To clarify the role of NCRIP in the regulation of this gene network, we further
208 analyzed their expression levels in three different comparisons: WTM vs. WTC, *r3b2* Δ C
209 vs. WTC, and *rdp1* Δ C vs. WTC (Fig 3B). Surprisingly, the differential expression in
210 both *rdp1* Δ and *r3b2* Δ mutants compared to the wild-type strain in saprophytic
211 conditions was almost identical to those found in the wild-type strain responding to
212 phagocytosis (Fig 3B). This analysis showed a group of genes activated both by
213 macrophage-mediated phagocytosis in the wild-type strain and by the lack of *rdp1* or
214 *r3b2* (Fig 3B, coincidences in red). This group of activated genes may correspond to
215 primary target genes repressed by NCRIP, suggesting a negative regulation of NCRIP in
216 the absence of macrophages that is released upon phagocytosis in the wild-type strain. A
217 second group consists of genes repressed both by the presence of macrophages in the
218 wild-type strain and the lack of *rdp1* or *r3b2* (Fig 3B, coincidences in blue). These genes
219 could be acting as secondary targets of the primary gene set.

220 Previous studies identified gene expression profiles during the phagocytosis of *M.*
221 *circinelloides*' wild-type spores [13]. Those profiles were validated by quantitative RT-
222 PCR using the following representative marker genes: *atf1*, *atf2*, *pps1*, and *aqp1*. These
223 marker genes showed a significant induction during macrophage phagocytosis and are
224 essential for this fungal pathogen to survive and cause infection. Our transcriptomic
225 analysis found that all of these marker genes were also controlled by NCRIP during
226 saprophytic growth (S1 Dataset), and thus, they were employed here to validate the
227 transcriptional pre-activation observed in the mutants *rdrp1Δ* and *r3b2Δ* without
228 macrophages (Fig 3C). We found that the four marker genes showed significant induction
229 in the two mutants without macrophages, similar to the previously reported increased
230 expression observed in the wildtype during phagocytosis [13], indicating that NCRIP
231 controls the response to phagocytosis by repressing it during non-stressful conditions.

232 Functional enrichment analysis of this gene set was performed to further
233 understand the biological processes controlled by this response (Fig 4). We observed a
234 clear alteration of the metabolism and transport that affects both carbohydrates and lipids
235 because the corresponding KOG classes included genes down- and upregulated. This was
236 associated with an overrepresentation of upregulated genes involved in amino acid
237 transport and metabolism, suggesting a metabolic change linked to the germination
238 process inside the phagosome. The harsh phagosomal environment might also be
239 responsible for the induction of genes related to the biosynthesis, transport, and
240 catabolism of secondary metabolites and extracellular structures to defend the fungus
241 from an oxidative challenge. Enrichment of downregulated genes involved in cell motility
242 and cytoskeletal processes could also be a part of a fungal strategy to produce competent
243 extracellular structures needed for survival and germination inside the phagosome. This

244 response was functionally similar to that observed from the whole gene profile identified
245 in the NCRIP mutants under non-stressful conditions (Fig 2).

246 **NCRIP negatively regulates the protective role of the canonical RNAi pathway in**
247 **the suppression of Grem-LINE1s retrotransposons**

248 The results presented above indicate that NCRIP represses genes during non-
249 stressful conditions and then releases its control upon phagocytosis by macrophages, a
250 clear challenging stimulus. However, the expression profiles analyzed did not reveal any
251 specific pathway involved in sporulation or mating. Because mutants in the machinery of
252 NCRIP also display defects in the production of zygospores during mating, we
253 hypothesized that NCRIP might also contribute in the regulation of genes involved in
254 other stresses such as antifungal agents [4,9] and genomic integrity stress, which could
255 alter complex cell processes involved in mating [10]. A recent study supported this
256 hypothesis, unveiling a direct link between the canonical RNAi pathway and the
257 protection of genome integrity against transposable elements in *M. circinelloides* [12].
258 The pericentric regions of *M. circinelloides* contain a large number of L1-like
259 retrotransposable elements of the Mucoromycotina species called Grem-LINE1s, which
260 are actively silenced by the canonical RNAi machinery. As suggested previously [9],
261 NCRIP might regulate the RNAi canonical core during the control of these transposable
262 elements by suppressing the epimutational pathway. In this sense, we characterized the
263 production of siRNAs from Grem-LINE1 transcripts in the pericentric regions of the
264 wild-type strains and the *r3b2Δ* and *rdrp1Δ* mutants (Fig 5A). The pericentric regions are
265 almost depleted of siRNAs in the *ago1Δ* and *dcl1 dcl2Δ* mutants, whereas the wild-type
266 strain exhibited an active production of siRNAs aligned to these loci, as previously
267 reported [12]. Interestingly, the *r3b2Δ* and *rdrp1Δ* mutants displayed an exaggerated
268 production of siRNAs compared to the wild-type strain, especially targeting the second

269 open reading frame (ORF2) and its reverse transcriptase domain (RVT). The over-
270 accumulation of siRNAs ($\geq 1.5 \log_2$ FC) is consistent among all Grem-LINE1s in the
271 *r3b2* Δ and *rdp1* Δ mutants (Fig 5B). These results suggest an enhanced activity of the
272 canonical RNAi machinery degrading the target retrotransposons when NCRIP is not
273 active and therefore, a negative regulatory role for NCRIP.

274 **Lack of NCRIP decreases virulence**

275 Virulence is a complex trait that depends on multiple genes and is controlled by
276 different biological processes [14]. The fact that NCRIP regulates the expression of
277 hundreds of genes involved in the response to phagocytosis suggests a role in controlling
278 critical pathways involved in virulence. To test this hypothesis, the *r3b2* Δ and *rdp1* Δ
279 mutants were used to perform survival assays in an immunosuppressed mouse model,
280 previously validated as a host model for infections with *M. circinelloides* [15]. The
281 survival rates were compared to those of mice injected with the wild-type virulent strain
282 R7B and the NRRL3631 strain, a natural soil isolate that served as an avirulent mock
283 control [16]. The results of these assays showed a significant reduction in virulence of the
284 two mutant strains (Log-rank Mantel-Cox test, $p = 0.0061$ in *rdp1* Δ vs. R7B; $p = 0.040$
285 in *r3b2* Δ vs. R7B; Fig 6), indicating that NCRIP controls the expression of genes involved
286 in virulence.

287

288 **Discussion**

289

290 Among the diversity of RNAi pathways in *M. circinelloides*, NCRIP is the most
291 recently discovered. It is a new mechanism that remains largely uncharacterized, and its
292 functional role in fungal physiology is the central unanswered question. Is it a non-
293 canonical RNAi degradation mechanism that clears and turns over damaged RNAs? Or
294 does it play a regulatory function controlling the expression of mRNAs at specific levels
295 depending on cellular requirements? The results obtained in this study unveiled a complex

296 regulatory role of NCRIP in fungal physiology rather than a simple degradation
297 mechanism for functional or damaged RNAs. Thus, we identified hundreds of genes
298 regulated by NCRIP, including genes involved in survival during phagocytosis. The
299 analysis of the spore response to the phagosome revealed a derepression of a complex
300 gene network activated in the fungal spore after the interaction with macrophages.
301 Moreover, we identified a negative regulatory role of NCRIP over the canonical RNAi
302 pathway in the control of transposable elements, extending the functional complexity of
303 this mechanism beyond the control of cellular mRNA levels. These complex functional
304 roles of NCRIP correlated with the pleiotropic phenotypes observed in mutants of this
305 pathway, including the reduced virulence described here.

306 Regarding the gene network regulated by NCRIP, previous studies suggested a
307 broad regulatory function of this pathway based on the discovery of 611 loci producing
308 sRNAs in a *dicer*-independent *rdrp*-dependent manner [10]. Here, we have directly
309 analyzed the transcriptomic profiles in NCRIP key mutants, identifying a substantial
310 number of DEGs in both *rdrp1* and *r3b2* mutants compared to the wild-type strain.
311 However, a significantly lower number of genes were regulated in these mutants upon
312 phagocytosis compared to the complex response observed in the wild-type strain [13].
313 The principal component analysis and the comparison of the four profiles among them
314 further confirmed this strong bias among samples. Thus, NCRIP showed a differential
315 regulatory intensity when rich and stressful environments were compared. These results
316 prompted us to hypothesize a repressive regulatory role of NCRIP under non-stress
317 conditions; hence, upon cellular challenges (like drug exposure or phagocytosis), the
318 repression would cease allowing the activation of the corresponding gene response.
319 Previous studies support this hypothesis, finding a similar regulatory mechanism in the
320 epimutational pathway, which also suggested a negative regulatory role under no stress

321 conditions [9]. A more in-depth analysis of the gene profiles and their expression levels
322 supports our hypothesis because the mutants activated the gene response to phagocytosis
323 before the interaction with macrophages. These findings might explain the augmented
324 oxidative stress resistance observed in the NCRIP mutants in vitro [10]. Thus, the mutants
325 in NCRIP could be unable to respond properly to stress because they show constitutive
326 expression of these genes which might lead to a pre-exposure adaptation to the stimuli.

327 Another regulatory role of NCRIP identified in this work unveiled a novel genetic
328 mechanism in which the canonical RNAi pathway and NCRIP work as antagonistic dual
329 machinery to control the movement of transposable elements. Previous studies reported
330 that most sRNAs produced by the NCRIP machinery map to exonic regions, whereas a
331 minimal amount of these sRNAs were found in intergenic regions and transposable
332 elements [10]. However, these studies were developed using initial annotation versions
333 of the *M. circinelloides* genome before the identification of the centromeric regions. Once
334 the centromeric regions were assembled, they were further characterized as rich in
335 repetitive sequences and Grem-LINE1 retrotransposons [12]. The expression of the
336 mobile elements is suppressed by the canonical RNAi machinery and correlates with an
337 abundant production of sRNAs and low mRNA levels. In this work, we found an
338 exacerbated production of sRNAs from centromeric transposons in the NCRIP mutants,
339 indicating an overactivation/derepression of the canonical RNAi pathway. These results
340 suggest a negative regulatory role of NCRIP over the canonical RNAi pathway,
341 analogous to the inhibitory role the NCRIP pathway exerts over the epimutational
342 pathway. Via the canonical RNAi machinery, the epimutational pathway silences target
343 genes to overcome growth inhibition caused by antifungal compounds and generates
344 epimutant strains that are resistant to drugs [3,4]. Conversely, the inactivation of NCRIP
345 leads to an overproduction of epimutant strains [9], suggesting either a competition

346 between NCRIP and the epimutational pathway for the transcripts of the target gene or
347 repression of NCRIP over the canonical mechanism. Here, we propose that the same
348 mechanism is operating in the control of the movement of pericentromeric
349 retrotransposons. This hypothesis explains why when the NCRIP is inactive most of the
350 retrotransposons are controlled by the canonical RNAi machinery and there is enhanced
351 production of sRNAs in *r3b2Δ* and *rdrp1Δ* mutants. Thus, both interacting RNAi
352 pathways could be essential for genome stability and integrity.

353 The role of the RNAi machinery in protecting genome integrity against the
354 movement of transposons is important during mating in several fungal models. In *C.*
355 *neoformans*, the mechanism of sex-induced silencing (SIS) defends the genome against
356 transposons during sexual development, whereas in several ascomycetes [17–19] an
357 RNAi mechanism operates to silence unpaired DNA in meiosis, including transposons
358 [20]. These surveillance mechanisms that protect genome integrity rely on the RNAi
359 canonical core, as in *M. circinelloides*. However, in this fungus, the canonical RNAi
360 pathway coexists with a regulatory mechanism based on NCRIP, which has not been
361 described in other fungal groups [10]. It is tempting to speculate that both the canonical
362 mechanism and NCRIP perform a fine control over retrotransposable movements to gain
363 genetic diversity in particular stressful conditions, allowing a transient activation of the
364 retrotransposons to overcome the insult. Alteration of this precise control may be
365 responsible for the defective mating observed in NCRIP mutants.

366 The pre-activated state of NCRIP mutants and their previously described oxidative
367 stress resistance suggests an advantage to resist the oxidative attack of macrophages.
368 However, our results showed a reduced pathogenic potential in both *rdrp1Δ* and *r3b2Δ*
369 mutants, indicating that NCRIP is necessary for virulence in Mucorales. These results
370 reveal that the resistance to oxidative stress in vitro did not improve the pathogenicity of

371 the mutants during in vivo interactions. The complex environment of the host during the
372 initial steps of phagocytosis could explain these results, because the fungus must respond
373 to phagosomal conditions, including oxidative stress, nutritional starvation, and pH
374 acidification. The intricate transcriptomic response displayed by the spores to counteract
375 the host was not fully replicated in the NCRIP mutants, and these mutants show DEGs
376 that are not regulated by phagocytosis (Fig 3A). On the other hand, the genetic
377 deregulation in NCRIP mutants might affect other fungal responses required during the
378 response to phagocytosis, or in further infection steps, such as tissue invasion, resulting
379 in a final negative balance for the fungal spore.

380 Our functional study unveiled a complex gene network conditionally regulated by
381 NCRIP. The analysis of this gene network revealed a remarkable function of NCRIP in
382 the negative regulation of the genetic response elicited during phagocytosis, suggesting
383 an essential role for this pathway in host-pathogen interactions. Altogether, the
384 identification of a large number of genes regulated by NCRIP and the subset involved in
385 the response to phagocytosis confirm the broad regulatory role of NCRIP, arguing against
386 a simpler role in clearance and turnover of RNAs. Instead, NCRIP emerges as a
387 mechanism controlling an extensive network of genes involved in different cellular
388 processes, with the capability of regulating them differentially after environmental
389 challenges that include antifungals agents, phagocytosis, and virulence. The role of
390 NCRIP controlling the genetic response to phagocytosis and the final phenotypic balance
391 impairing virulence are new contributions to understanding the difficult to treat and
392 challenging to manage infection of mucormycosis.

393 **Materials and Methods**

394 **Fungal strains, cell cultures, and RNA purification.**

395 The fungal strains used in this work derived from *M. circinelloides* f. *lusitanicus*
396 CBS277.49. The wild-type control strain for the RNA-seq analysis and virulence assays
397 is R7B [21]. The strains defective in the NCRIP are MU419 (*rdrp1*Δ) [22] and MU412
398 (*r3b2*Δ) [10]. All the strains compared for the gene expression analysis were auxotroph
399 for leucine. The strain NRRL3631 was used as an avirulent control for the mice infection
400 experiments [23]. *M. circinelloides* cultures were grown in rich media YPG pH 4.5 at
401 26°C for optimal growth and sporulation. Spores were harvested and filtered using a
402 Falcon® 70 µm cell strainer before confronting with macrophages or animal models.

403 The host-pathogen interactions were performed confronting spores from R7B, MU419,
404 and MU412 with mouse macrophages (cell line J774A.1; ATCC TIB-67) in a ratio 1.5:1
405 (spores:macrophages) following the protocol described in [13]. In summary, the
406 interactions were maintained at 37°C in L15 medium (Capricorn Scientific) supplemented
407 with 20% of Fetal Bovine Serum (Capricorn Scientific) for 5 hours, ensuring all the spores
408 were phagocytosed. For saprophytic conditions, the same concentration of spores was
409 cultured in L15 medium as described.

410 For RNA purification, two replicates of each sample were pooled, and RNA was extracted
411 using the RNeasy plant minikit (Qiagen, Hilden, Germany), following the manufacturer
412 procedure.

413 **RNA-sequencing analysis for gene expression and small RNA production**

414 Raw datasets were quality-checked using FASTQC v0.11.8 before and after removing
415 adapter and contaminant sequences with Trim Galore! v.0.6.2
416 (<http://www.bioinformatics.babraham.ac.uk/projects/>). Messenger RNA reads were
417 aligned to the *M. circinelloides* f. *lusitanicus* v2.0 genome (Mucci2 [24]) using STAR
418 v.2.7.1a [25] and the subsequent Binary Alignment Maps (BAM) were used to create
419 individual count matrices with HTSeq v.0.9.1 [26], excluding multi-mapping reads.

420 Differential gene expression and principal components analysis (PCA) were performed
421 by *limma* package v.3.38.3 [27]; genes above a reliable threshold used in a previous study
422 [13] (False Discovery rate [FDR] ≤ 0.05 ; \log_2 fold change [\log_2 FC] ≥ 1.0 ; and average
423 count per million reads (CPM) ≥ 1.0) were considered differentially expressed genes
424 (DEGs) and used for downstream analyses unless more stringent criteria is stated. DEGs
425 were classified according to Eukaryotic Orthologous Groups (KOG) and Gene Ontology
426 (GO) terms using EggNOG-mapper v2.0 [28,29] to perform KOG class enrichment
427 analyses with KOGMWU package v.1.2 [30]; statistical significance was assessed with a
428 Fisher's exact test (p-value ≤ 0.05). Small RNA reads were obtained from previous studies
429 (see Data Availability) and aligned to the *M. circinelloides* f. *lusitanicus* MU402 v1.0
430 genome (Muccir1_3 [12]) using the Burrows-Wheeler Aligner (BWA) v.0.7.8 [31]. This
431 genome was PacBio-sequenced using long reads and thus, exhibit a greater content of
432 repeated elements [12]. The number of overlapping aligned reads per 25-bp bin was used
433 as a measure of coverage, obtaining Coverage was normalized to bins per million reads
434 (BPM) in 25-bp bins with deepTools v3.2.1 [32] bamCoverage function. The resulting
435 bigWig files were visualized with the deepTools pyGenomeTracks module using the
436 centromeric and transposable element annotations found by Navarro-Mendoza et al. 2019
437 [12].

438 **RT-qPCR quantification**

439 Replicate samples for the host-pathogen interactions and control conditions were used for
440 RT-qPCR analysis. Once the mRNA was purified and treated with TURBO DNase
441 (Thermo Fisher), the cDNA was synthesized from 1 μ g of total RNA using the iScript
442 cDNA synthesis kit (Bio-Rad). The RT-qPCR was performed in triplicate using 2X
443 SYBR green PCR master mix (Applied Biosystems) with a QuantStudio TM 5 flex
444 system (Applied Biosystems) using 2X SYBR green PCR master mix (Applied

445 Biosystems) following the supplier's recommendations. To ensure non-specific
446 amplification, non-template control and melting curve were tested. The primer sequences
447 used for the quantification of genes *atf1*, *atf2*, *pps1*, *aqp1*, and rRNA *18S* are listed in S1
448 Table. The efficiencies of every pair of primers were approximately identical; thus the
449 relative gene expression of the target genes was obtained the delta-delta cycle threshold
450 ($\Delta\Delta C_t$) method, normalizing for the endogenous control rRNA *18S*.

451 **Virulence assays**

452 The murine infection assays for Mucorales virulence were performed using OF-1 male
453 mice weighing 30g (Charles River, Barcelona, Spain) [13,23,33]. The mice were
454 immunosuppressed with the administration of cyclophosphamide (200 mg/kg of body
455 weight) via intraperitoneal, 2 days prior to infection and once every 5 days thereafter.
456 Groups of 10 mice were challenged with 1×10^6 spores of the strains R7B, NRRL3631,
457 MU419 and MU412. The infections were performed intravenously via retroorbital
458 injection following the protocol described by Chang et al. 2019 [5]. Before the injection,
459 mice were anesthetized by inhalation of isoflurane, and then the animals were visually
460 monitored while recovering from the anesthesia. Mice were housed under established
461 conditions with free food and autoclaved water. The animal welfare was checked twice
462 daily for 20 days, and those following the criteria for discomfort were euthanized by CO₂
463 inhalation. The significance of survival rates was quantified using the Kaplan-Meier
464 estimator (Graph Pad Prism). Differences were considered statistically significant at a p-
465 value of ≤ 0.05 in a Mantel-Cox test.

466 **Data availability**

467 The raw data and processed files generated by this work are deposited at the Gene
468 Expression Omnibus (GEO) repository and are publicly available through the project
469 accession number GSE142543. These data were compared to a wild-type strain in the

470 same conditions, previously available at GEO [13] under the following sample accession
471 numbers: GSM3293661 and GSM3293662 (wild-type strain single-cultured); and
472 GSM3293663 and GSM3293664 (wild-type strain co-cultured with mouse
473 macrophages). Mucci2 [24] and Muccir1_3 [12] genomes and annotation files can be
474 accessed at the Joint Genome Institute (JGI) website (<http://genome.jgi.doe.gov/>) and
475 used under the JGI Data Usage Policy. The small RNA raw data were available to the
476 public through the following NCBI SRA run accession numbers: SRR039123 (wild-type
477 strain) [34], SRR836082 (*ago1*Δ mutant strain) [35], SRR039128 (double *dcl1*Δ *dcl2*Δ
478 mutant strain) [34], SRR039126 (*rdrp1*Δ mutant strain) [34], and SRR1576768 (*r3b2*Δ
479 mutant strain) [10].

480 **Ethics statement**

481 To guarantee the welfare of the animals and the ethics of any procedure related to animal
482 experimentation all the experiments performed in this work complied with the Guidelines
483 of the European Union Council (Directive 2010/63/EU) and the Spanish RD 53/2013.
484 Experiments and procedures were supervised and approved by the University of Murcia
485 Animal Welfare and Ethics Committee and the Council of Water, Agriculture, Farming,
486 Fishing and Environment of Murcia (Consejería de Agua, Agricultura, Ganadería, Pesca
487 y Medio Ambiente de la CARM), Spain (authorization number REGA
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615

616 **Supporting information**

617 **S1 Dataset. Differentially expressed genes in NCRIP mutants.**

618 **S1 Table. Primers used in the study.**

619

620 **Figure captions**

621 **Figure 1. NCRIP regulates a vast gene network via the cooperation of R3B2 and**

622 **RdRP1. (A)** Principal component (PC) analysis biplot of gene abundances (in counts per

623 million [CPM], mean CPM > 1.0), showing the similarity across the color- and shape-

624 coded samples. **(B)** Venn diagram of significant differentially expressed genes (DEGs,

625 $\log_2FC \geq |1.0|$, $FDR \leq 0.05$) in the depicted mutant strains and conditions compared with

626 the wild-type strain in the same condition. DEGs overlap if they share a similar expression

627 pattern (either up- or downregulation).

628 **Figure 2. NCRIP regulates key functional categories involved in saprophytic growth.**

629 Enrichment analysis of DEGs in each Eukaryotic Orthologous Groups (KOG) class.

630 Significant enrichments (Fisher's exact test, $P \leq 0.05$) in a given mutant strain and

631 condition compared to the wild-type strain is shown as an uplifted rectangle. A measure

632 of up- (red) or downregulation (blue) of each KOG class is represented as a colored scale

633 of delta-rank values (the difference between the mean differential expression value of all

634 genes in each KOG class and the mean differential expression value of all other genes).

635 KOG classes and experimental conditions (mutant strains and presence/absence of

636 macrophages) are clustered according to the similarity of their delta rank values.

637 **Figure 3. NCRIP controls the response to macrophage phagocytosis by inhibiting its**

638 **targets under non-stressful conditions. (A)** Venn diagram of DEGs ($\log_2FC \geq |1.5|$,

639 $FDR \leq 0.05$) in the wild-type, *r3b2* Δ , and *rdrp1* Δ mutant strains phagocytosed by

640 macrophages compared with their non-phagocytosed controls. DEGs overlap if they share

641 a similar expression pattern (either up- or downregulation). **(B)** Heatmap of the 908 DEGs

642 found in (A) that were responding to phagocytosis in the wild-type strain but were not

643 differentially expressed in either NCRIP mutant strain. Genes and experimental
644 conditions are clustered by similarity to compare the response to phagocytosis in the wild-
645 type strain with the response of the NCRIP mutants in non-stressful conditions. DEGs are
646 color-coded to depict the degree of upregulation (red) or downregulation (blue) in each
647 condition. (C) Bar plot of *atf1*, *atf2*, *pps1* and *aqp1* expression differences in R3B2Δ, and
648 RdRP1Δ mutant strains compared with the wild-type strain in non-stressful conditions,
649 i.e. incubation in cell-culture medium without macrophages for 5 hours. Log₂ fold-change
650 differential expression levels were quantified by RT-qPCR and normalized using *rRNA*
651 18S as an internal control. Error bars correspond to the SD of technical triplicates and
652 significant differences are denoted by asterisks (* for $P \leq 0.05$, ** for $P \leq 0.005$, and ***
653 for $P < 0.0001$ in an unpaired t-test).

654 **Figure 4. Functional analysis of the response controlled by NCRIP during**
655 **macrophage interaction.** Enrichment analysis of the 908 DEGs controlled by NCRIP,
656 showing up- (red) and downregulated (blue) genes. Fold-enrichment in each KOG
657 category is plotted and significant enrichments (Fisher's exact test, $P \leq 0.05$) is marked
658 by an asterisk (*).

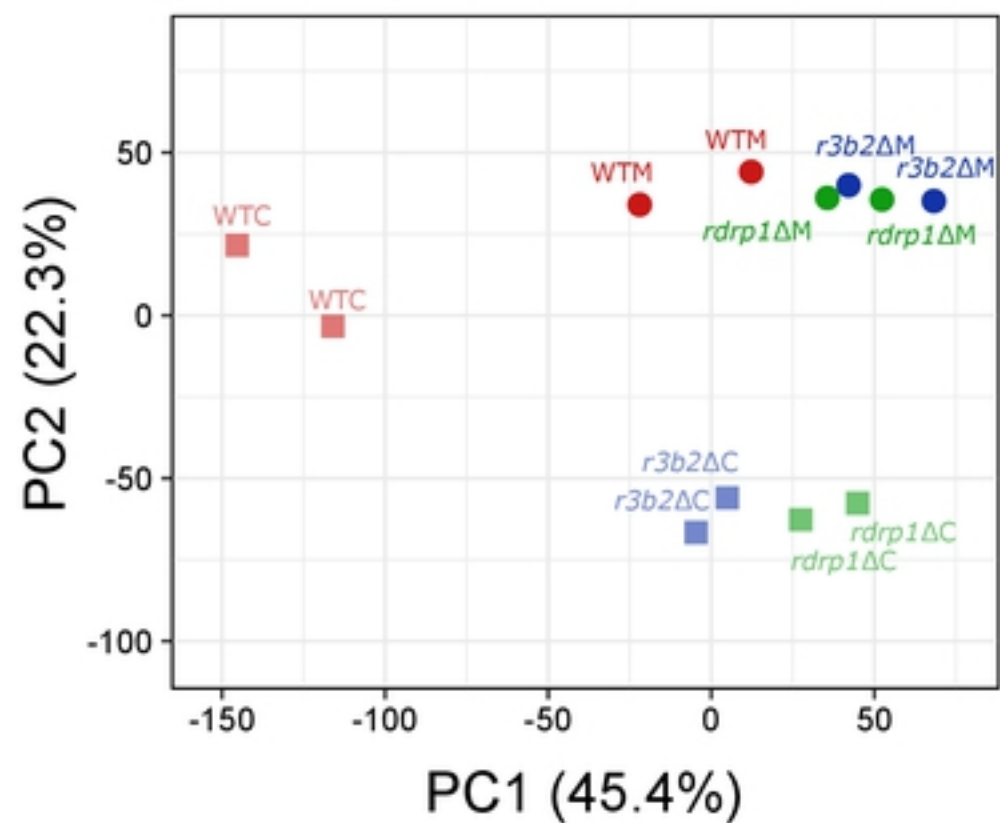
659 **Figure 5. NCRIP competes with the epimutational pathway to regulate transposable**
660 **elements. (A)** Genomic view of centromeric chromatin (*CEN4*) displaying the
661 kinetochore-binding region enrichment that marks the centromere (*CEN*, blue),
662 annotation of transposable elements (colored blocks), and transcriptomic data of sRNAs
663 (red) in *M. circinelloides* wild-type, epimutational pathway (*ago1Δ*, double *dcl1Δ/dcl2Δ*)
664 and NCRIP (*rdrp1Δ* and *r3b2Δ*) deletion mutant strains after 48 h of growth in rich
665 medium. The zoom below represents a single Grem-LINE1 and the NCRIP regulation by
666 small RNAs. Open reading frames (ORF1 in green arrows and ORF2 in red arrows) and
667 protein domains predicted from their coding sequences are shown as colored blocks (zf-

668 RVT, zinc-binding in reverse transcriptase [PF13966]; RVT, reverse transcriptase
669 [PF00078]; AP, AP endonuclease [PTHR22748]; and ZF, zinc finger [PF00098 and
670 PF16588]). **(B)** Heatmap of the differential expression of Genomic retrotransposable
671 elements of Mucoromycotina LINE1-like (Grem-LINE1s) in the depicted RNAi mutants
672 compared to the RNAi-proficient wild-type strain. Grem-LINE1s are numbered
673 according to Navarro et al. classification [12].

674 **Figure 6. NCRIP is involved in mucormycosis.** The virulence of *r3b2Δ* and *rdrp1Δ*
675 mutant strains was assessed in a survival assay using immunosuppressed mice as a
676 mucormycosis model. Groups of ten mice were infected intravenously with 1×10^6 spores
677 from each strain (color-coded). Survival rates were statistically analyzed for significant
678 differences (* for $P \leq 0.05$ in a Mantel-Cox test) compared with a virulent control strain.
679 NRRL3631 was used as an avirulent mock control of infection.

Figure 1

A



B

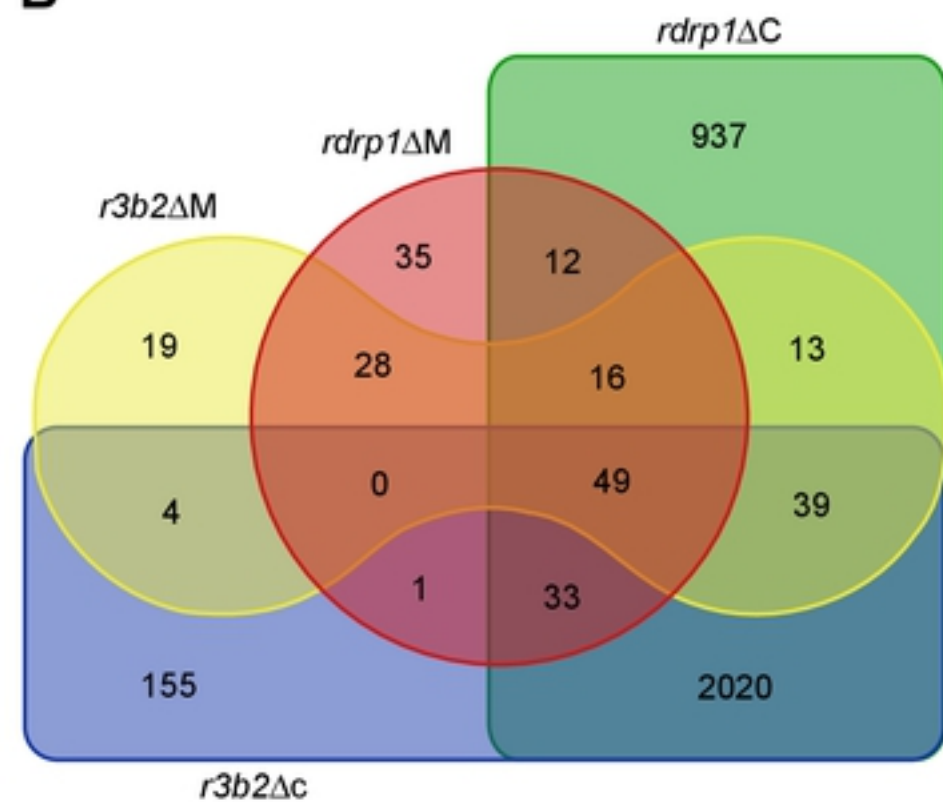
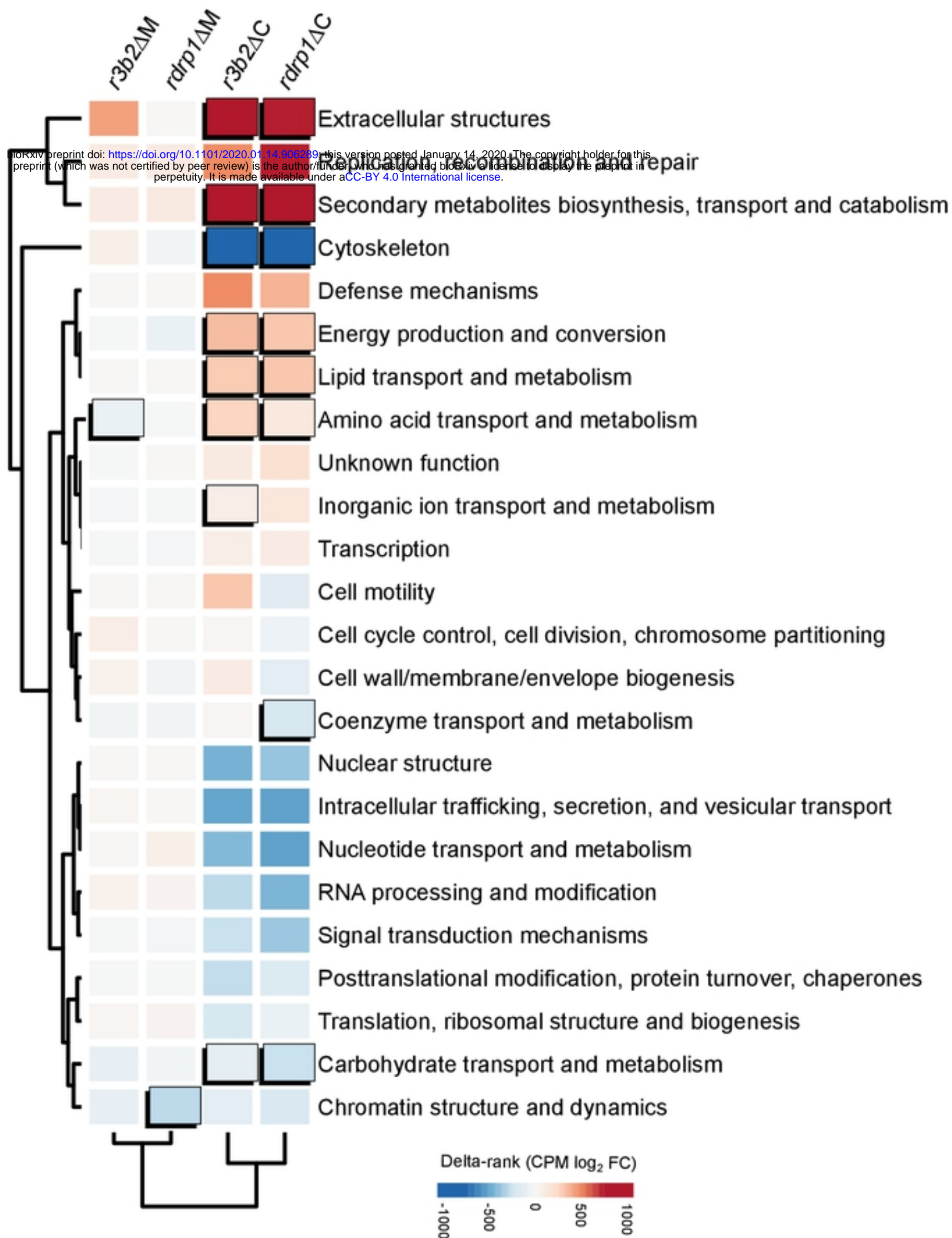


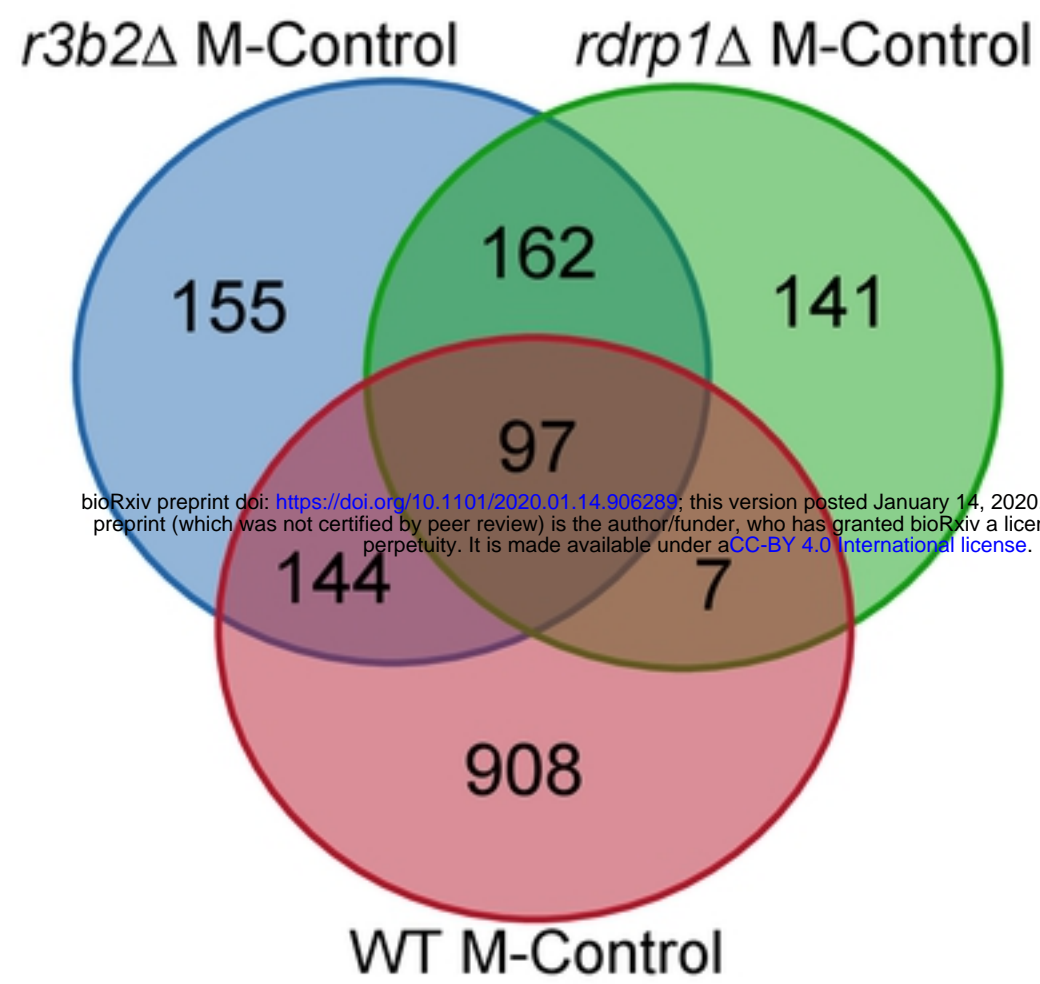
Figure 2



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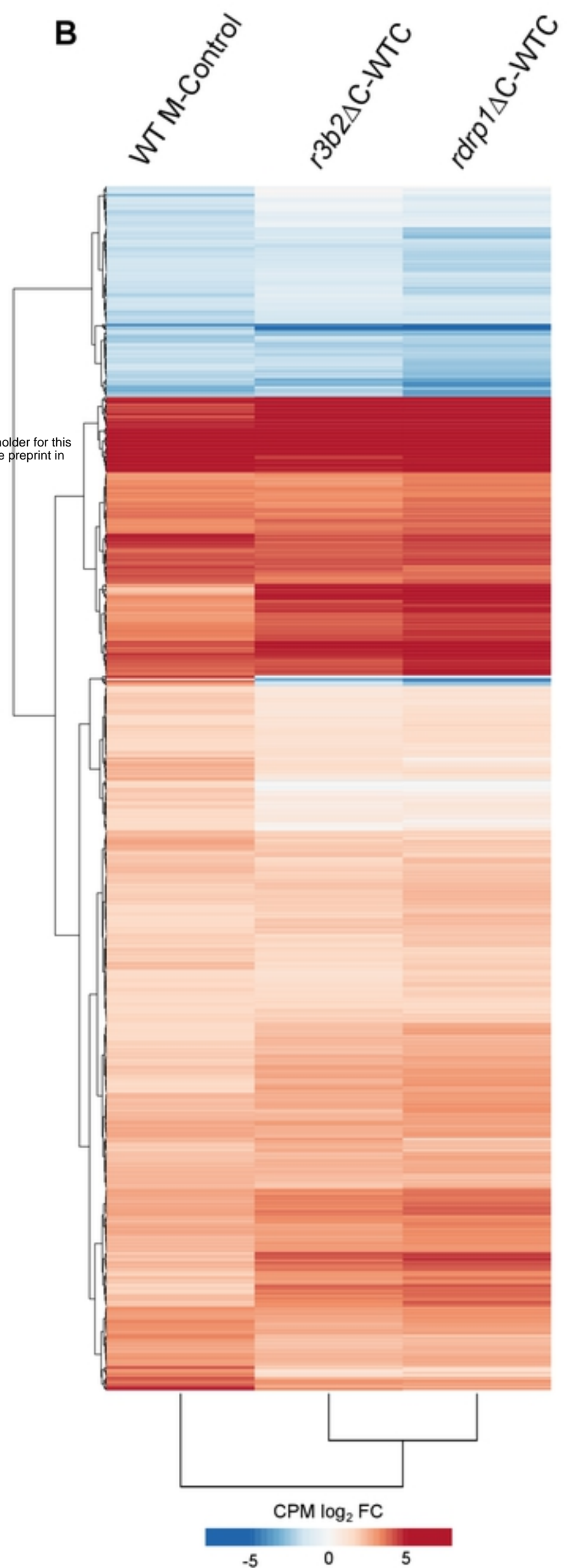
Figure 3

A



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B



C

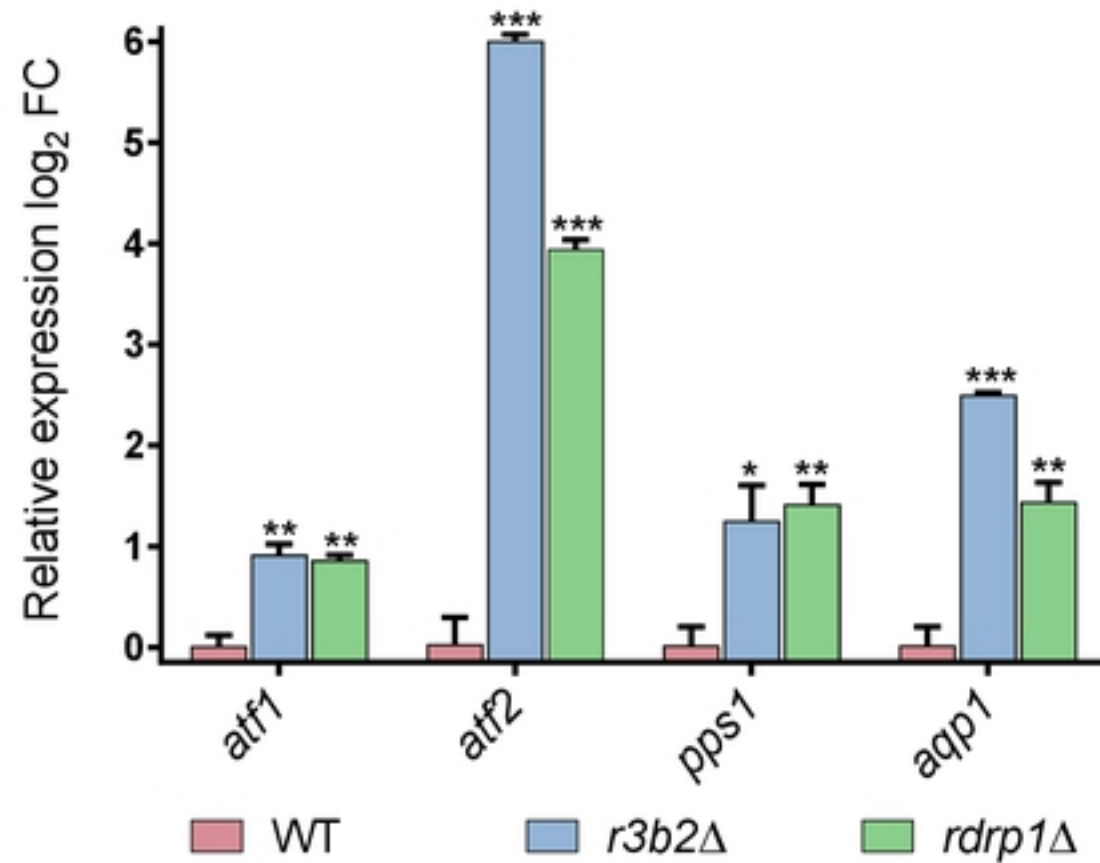


Figure 4

Amino acid transport and metabolism
Carbohydrate transport and metabolism
Cell cycle control, cell division, chromosome partitioning
Cell motility
Cell wall/membrane/envelope biogenesis
Chromatin structure and dynamics
Coenzyme transport and metabolism
Cytoskeleton
Defense mechanisms
Energy production and conversion
Extracellular structures
Inorganic ion transport and metabolism
Intracellular trafficking, secretion, and vesicular transport
Lipid transport and metabolism
Nuclear structure
Nucleotide transport and metabolism
PTM, protein turnover, chaperones
Replication, recombination and repair
RNA processing and modification
SM biosynthesis, transport and catabolism
Signal transduction mechanisms
Transcription
Translation, ribosomal structure and biogenesis
Function unknown

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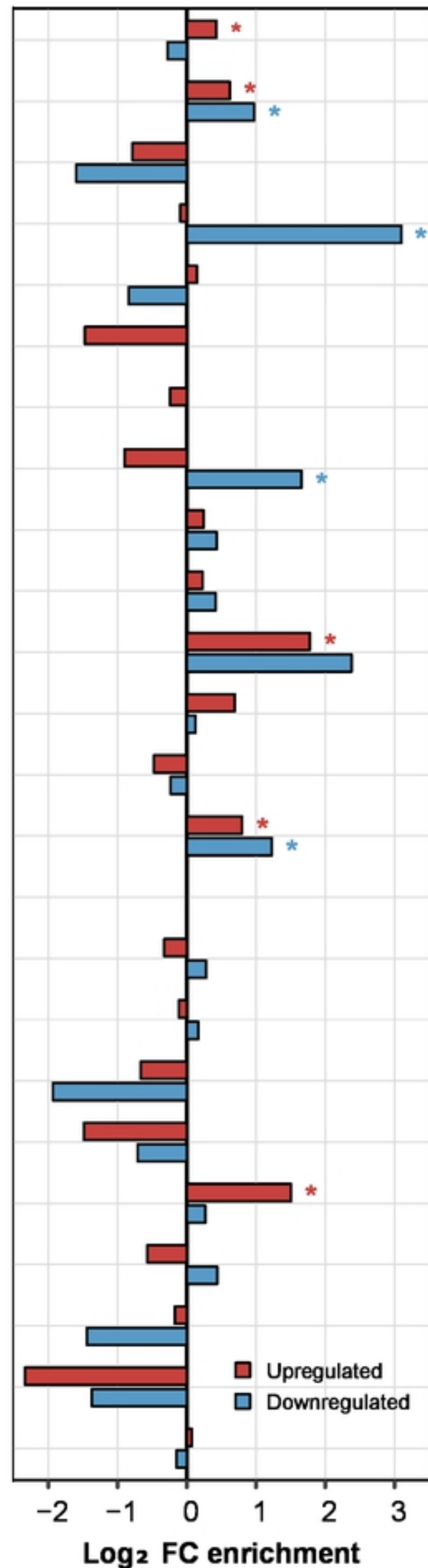
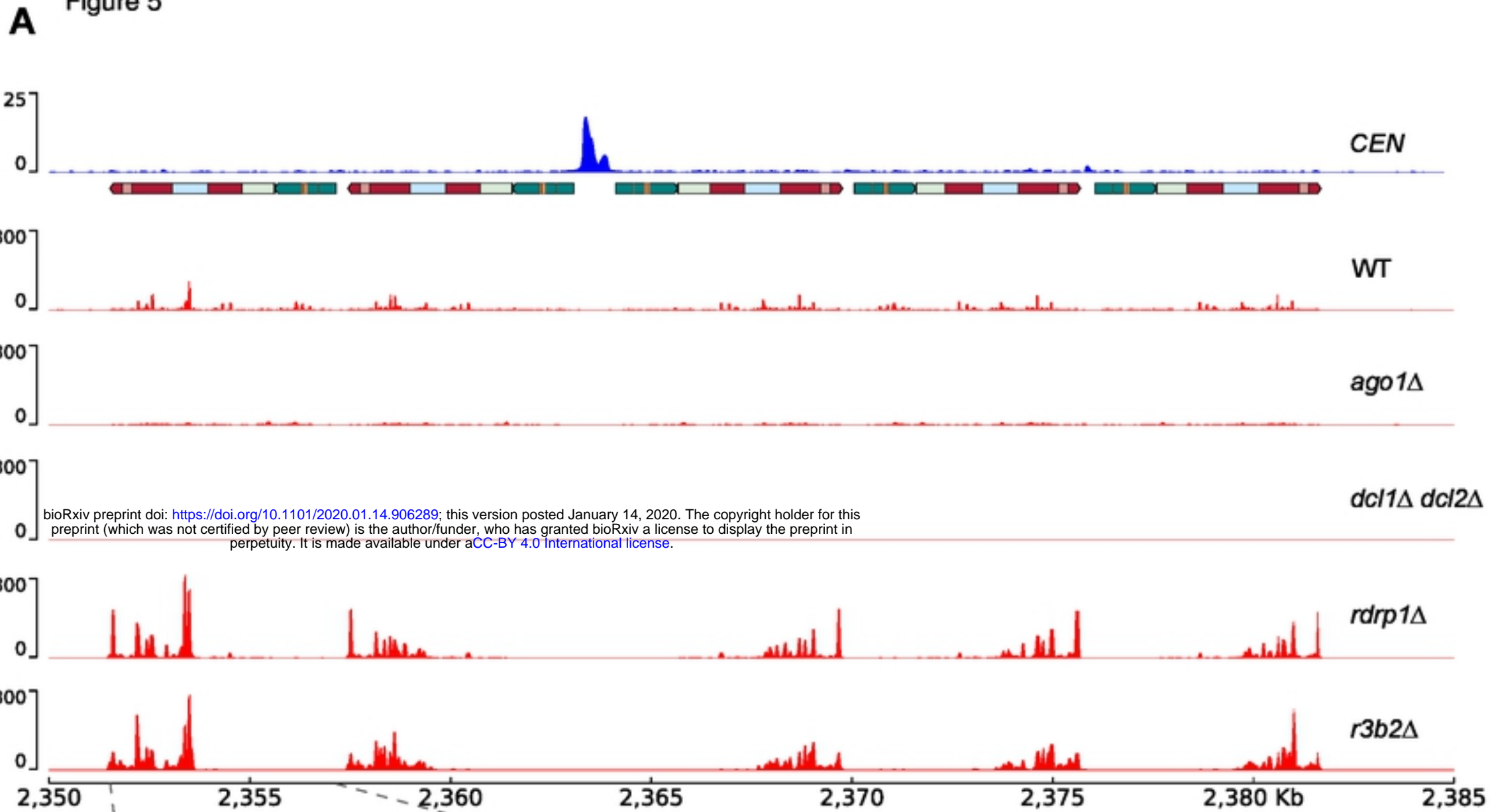


Figure 5



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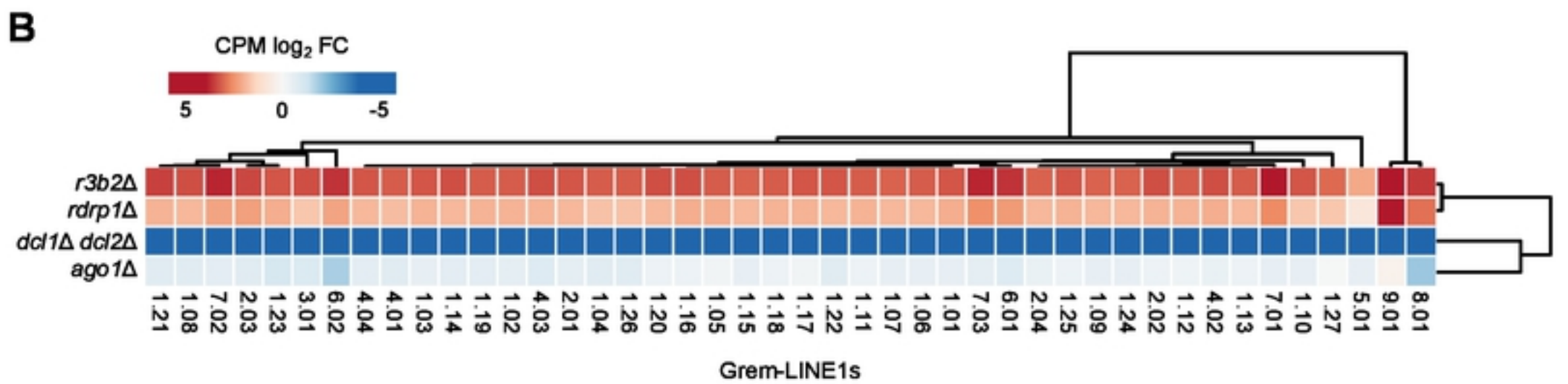
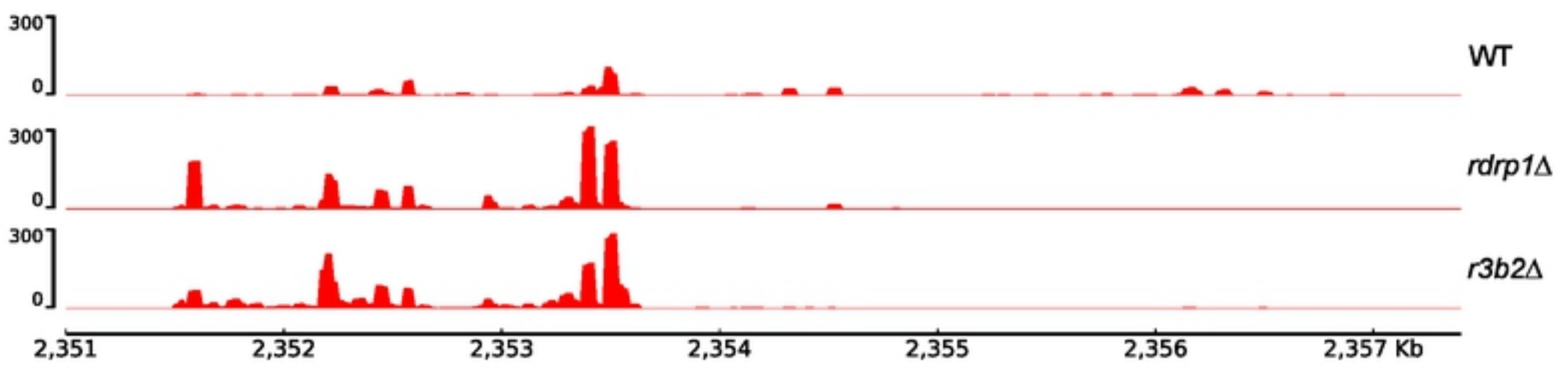


Figure 6

