

Investigating the effect of Target of Rapamycin kinase inhibition on the *Chlamydomonas reinhardtii* phosphoproteome: from known homologs to new targets

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1 Summary

- 2 • Target of Rapamycin (TOR) kinase is a conserved regulator of cell growth whose activity is
3 modulated in response to nutrients, energy and stress. Key proteins involved in the pathway
4 are conserved in the model photosynthetic microalga *Chlamydomonas reinhardtii*, but the
5 substrates of TOR kinase and downstream signaling network have not been elucidated. Our
6 study provides a new resource for investigating the phosphorylation networks governed by the
7 TOR kinase pathway in *Chlamydomonas*.
- 8 • We used quantitative phosphoproteomics to investigate the effects of inhibiting
9 *Chlamydomonas* TOR kinase on dynamic protein phosphorylation. Wild-type and AZD-
10 insensitive *Chlamydomonas* strains were treated with TOR-specific chemical inhibitors
11 (rapamycin, AZD8055 and Torin1), after which differentially affected phosphosites were
12 identified.
- 13 • Our quantitative phosphoproteomic dataset comprised 2,547 unique phosphosites from 1,432
14 different proteins. Inhibition of TOR kinase caused significant quantitative changes in
15 phosphorylation at 258 phosphosites, from 219 unique phosphopeptides.
- 16 • Our results include *Chlamydomonas* homologs of TOR signaling-related proteins, including a
17 site on RPS6 with a decrease in phosphorylation. Additionally, phosphosites on proteins
18 involved in translation and carotenoid biosynthesis were identified. Follow-up experiments
19 guided by these phosphoproteomic findings in lycopene beta/epsilon cyclase showed that
20 carotenoid levels are affected by TORC1 inhibition and carotenoid production is under TOR
21 control in algae.

22 Keywords: Phosphoproteomics, *Chlamydomonas*, AZD8055, rapamycin, Torin1, target of
23 rapamycin, TOR, NL

24 **Introduction**

25 The Target of Rapamycin (TOR) protein kinase is a conserved eukaryotic growth regulator whose
26 activity is modulated in response to stress, nutrients and energy supply (Wullschleger *et al.*, 2006;
27 Loewith & Hall, 2011; Dobrenel *et al.*, 2016a; González & Hall, 2017; Pérez-Pérez *et al.*, 2017).
28 In metazoans and fungi, TOR is found in two compositionally and functionally distinct
29 multiprotein complexes (TORC1) and (TORC2) that control rates of biosynthetic growth and
30 cytoskeletal dynamics respectively (Raught *et al.*, 2001; Wullschleger *et al.*, 2006). In the green
31 lineage (algae and land plants), only homologs of TORC1 proteins have been identified (Diaz-
32 Troya *et al.*, 2008; van Dam *et al.*, 2011; Dobrenel *et al.*, 2016a). TORC1 kinase activity is
33 modulated by nutrients and stress, and serves to control protein biosynthesis and other metabolic
34 processes in response to environmental conditions (Raught *et al.*, 2001). Selective chemical
35 inhibitors of TOR kinase including rapamycin, AZD8055, and Torin1 have been instrumental in
36 dissecting the TOR signaling pathway (Fingar & Blenis, 2004; Thoreen *et al.*, 2009; Chresta *et al.*,
37 2010; Benjamin *et al.*, 2011). Rapamycin (Rap) inhibits TORC1 activity through an allosteric
38 mechanism requiring formation of a FKBP12-Rap complex (Heitman *et al.*, 1991; Brown *et al.*,
39 1994; Sabatini *et al.*, 1994). Recent studies support the notion that several functions of TOR kinase
40 are not inhibited by rapamycin (Thoreen *et al.*, 2009). Instead, novel drugs like Torin1 and
41 AZD8055 have been reported to more completely inhibit TOR kinase by acting as ATP-
42 competitors (Thoreen *et al.*, 2009; Chresta *et al.*, 2010). Torin1 has slower off-binding kinetics
43 than other mTOR inhibitors in mammalian cell lines, possibly due to conformational change
44 induction in the kinase that is energetically more difficult to recover from leading to a more
45 pronounced and longer inhibition of the TORC1 pathway (Liu *et al.*, 2013). AZD8055 is an ATP-
46 competitive inhibitor of mTOR and all PI3K class I isoforms noted to inhibit the mTORC1 and
47 mTORC2 substrate phosphorylation (Roohi & Hojjat-Farsangi, 2017). These drugs were used to
48 inhibit TOR activity in plants where rapamycin treatment is not highly effective (Zhang *et al.*,
49 2011; Montane & Menand, 2013).

50 The role of TOR in mammalian and fungal cell metabolism has been extensively investigated
51 (Wullschleger *et al.*, 2006; Dibble & Manning, 2013; Saxton & Sabatini, 2017), while its role in
52 photosynthetic eukaryotes is less well established (Zhang *et al.*, 2013; Xiong & Sheen, 2014;
53 Dobrenel *et al.*, 2016a). TOR has been shown to control growth, metabolism and life span in the

54 model plant *Arabidopsis thaliana* (*Arabidopsis*) (Dobrenel *et al.*, 2011; Ren *et al.*, 2012; Xiong,
55 Y. & Sheen, J., 2012; Xiong *et al.*, 2013) where the TOR gene is essential (Menand *et al.*, 2002).
56 The model green alga *Chlamydomonas reinhardtii* (*Chlamydomonas*) has key TORC1 complex
57 proteins encoded by single-copy genes including TOR (Cre09.g400553.t1.1), regulatory associate
58 protein target of rapamycin (RAPTOR) (Cre08.g371957.t1.1), and lethal with sec-13 protein 8
59 (LST8) (Cre17.g713900.t1.2) (Diaz-Troya *et al.*, 2008; van Dam *et al.*, 2011). Treatment of
60 *Chlamydomonas* cultures with rapamycin has been shown to slow but not completely arrest cell
61 growth (Crespo *et al.*, 2005), activate autophagy (Perez-Perez *et al.*, 2010), and induce lipid droplet
62 formation (Imamura *et al.*, 2015; Rodrigues *et al.*, 2015). Recent work reported a connection
63 between TOR kinase and inositol polyphosphate signaling that governs carbon metabolism and
64 lipid accumulation (Couso *et al.*, 2016). *Chlamydomonas* cells are sensitive to Torin1 and
65 AZD8055 that are potent inhibitors of cell growth at saturating doses (Couso *et al.*, 2016) and
66 induce triacylglycerol accumulation (Imamura *et al.*, 2016). However, the TOR pathway in
67 *Chlamydomonas* has yet to be extensively characterized and, to date, only a limited number of
68 candidate TOR kinase substrates have been identified.

69 We characterized the phosphoproteome of *Chlamydomonas* that produced a conservative estimate
70 of 4,588 phosphoproteins / 15,862 unique phosphosites (Wang *et al.*, 2014) through a qualitative
71 strategy involving extensive fractionation and complementary enrichment strategies, and have
72 now developed label-free quantification (LFQ) to allow simultaneous quantification of 2,547
73 *Chlamydomonas* phosphosites (Werth *et al.*, 2017). Herein we characterized the effects of TOR
74 inhibition on the *Chlamydomonas* phosphoproteome. Cultures treated with saturating doses of
75 different TOR inhibitors (rapamycin, AZD8055 and Torin1) revealed hundreds of affected
76 phosphosites with a significant overlap observed between those seen with different inhibitors.
77 Phosphosites from an AZD-resistant mutant were compared with wild type after AZD treatment
78 revealing very few potential off target effects. Hierarchical clustering was used to classify sites
79 and motif analysis was used to assess consensus motifs in clusters.

80 **Materials and Methods**

81 *Cell culturing and drug treatment.*

82 Strain CC-1690 wild-type mt⁺ (Sager 21 gr) (Sager, 1955) was used for the wild-type
83 Chlamydomonas analysis across all chemical inhibitors. For the control AZD-insensitive strain
84 experiments, strain was obtained from the Umen laboratory (Donald Danforth Plant Science
85 Center). All cultures were maintained on TAP (Tris acetate phosphate) agar plates and grown in
86 350-mL TAP liquid cultures at 25°C as previously described (Couso *et al.*, 2016). Experiments
87 were done using five replicate cultures grown to exponential phase ($1-2 \times 10^6$ cells/mL) for each
88 drug condition and control and quenched with 40% methanol prior to harvesting by centrifuging
89 at 4000 g for 5 min and discarding supernatant. To limit batch effects, replicate “n” of each drug
90 and control were harvested together (Figure 1) prior to downstream processing. Cell pellets were
91 then flash frozen using liquid nitrogen and stored at -80°C until use. For AZD8055-, Torin 1-, and
92 rapamycin- treated (LC Laboratories) cultures, drug was added to a final concentration of 500 nM
93 for rapamycin and Torin 1, and 700 nM for AZD8055 from 1mM stocks in DMSO for 15 min
94 prior to harvesting. For control replicates, just drug vehicle (DMSO) without a chemical inhibitor
95 was added to each replicate culture for 15 min prior to harvesting.

96 *Protein extraction.*

97 Cell pellets were resuspended in lysis buffer containing 100 mM Tris, pH 8.0 with 1x
98 concentrations of cOmplete protease inhibitor and phosSTOP phosphatase inhibitor cocktails
99 (Roche, Indianapolis, IN, USA). Cells were lysed via sonication using an E220 focused
100 ultrasonicator (Covaris, Woburn, MA, USA) for 120 s at 200 cycles/burst, 100 W power and 13%
101 duty cycle. Following ultrasonication, the supernatant was collected from cellular debris by
102 centrifugation for 10 min at 15,000 g at 4°C and proteins were precipitated using 5 volumes of
103 cold 100 mM ammonium acetate in methanol. Following 3 hr incubation at -80°C, protein was
104 pelleted by centrifugation for 5 min at 2,000 g followed by two washes with fresh 100 mM
105 ammonium acetate in methanol and a final wash with 70% ethanol. Cell pellets were resuspended
106 in 8M urea and protein concentration was determined using the CB-X assay (G-Biosciences, St.
107 Louis, MO, USA).

108

109 *Protein digestion and reduction.*

110 Samples were reduced using 10 mM dithiothreitol for 30 min at RT and subsequently alkylated
111 with 40 mM iodoacetamide for 45 min in darkness at RT prior to overnight digestion. Samples
112 were diluted 5-fold in 100 mM Tris following alkylation and digestion was performed at 25C for
113 16 h with Trypsin Gold (Promega) at a protease:protein ratio of 1:50.

114 *Solid-phase extraction.*

115 After digestion, samples were acidified to pH<3.0 with trifluoroacetic acid (TFA). Pelleted,
116 undigested protein was cleared from the supernatant by centrifugation for 5 min at 5,000 g prior
117 to solid-phase extraction. Desalting was performed using C18 50 mg Sep-Pak cartridges (Waters).
118 Columns were prepared by washing with acetonitrile (MeCN) followed by 80%
119 MeCN/20% H_2O /0.1% TFA and 0.1% TFA. Digested protein lysates were applied to the columns
120 and reloaded twice before being washed with 0.1% TFA and eluted using 80%
121 MeCN/20% H_2O /0.1% TFA.

122 *Phosphopeptide enrichment and clean-up.*

123 Following protein digestion and solid-phase extraction, replicates were dried down using vacuum
124 centrifugation and phosphopeptide enrichment was performed on 2-mg aliquots of each sample
125 using 3 mg Titansphere Phos- TiO_2 kit spin columns (GL Sciences) as previously described (Werth
126 *et al.*, 2017). After enrichment, samples were dried down and desalted again using ZipTips
127 (Millipore) as per manufacturers protocol prior to LC-MS/MS acquisition.

128 *LC-MS/MS acquisition and data processing.*

129 Following ZipTip clean-up, peptides were dried down and resuspended in 20 μ L of 0.1% TFA,
130 5% MeCN before separation via a 90-min linear gradient from 95% H_2O /5% MeCN/0.1% formic
131 acid (FA) to 65% H_2O /35% MeCN/0.1% FA via a NanoAcquity UPLC (Waters) using a C18
132 column (NanoAcquity UPLC 1.8 μ m HSS T3, 75 μ m \times 250 mm). A TripleTOF 5600 (AB Sciex)
133 Q-TOF was operated in positive-ionization nanoelectrospray and high-sensitivity mode for data
134 acquisition as previously described (Slade *et al.*, 2015). In addition to the Supporting Information
135 tables for MS datasets, the mass spectrometry proteomics data have been deposited to the
136 ProteomeXChange Consortium via PRIDE partner repository(Vizcaíno *et al.*, 2013) identifier

137 PXD007221. Acquired spectra (*.wiff) files were imported into Progenesis QI for proteomics
138 (v2.0, Nonlinear Dynamics) as previously described (Werth *et al.*, 2017) with peptide sequence
139 determination and protein inference done by Mascot (v.2.5.1; Matrix Science) using the *C.*
140 *reinhardtii* Phytozome v.11 database (www.phytozome.net/; accessed May 2015) appended with
141 the NCBI chloroplast and mitochondrial databases (19,603 entries) and sequences for common
142 laboratory contaminants (<http://thegpm.org/cRAP/>; 116 entries). For database searching, trypsin
143 protease specificity with up to two missed cleavages, peptide/fragment mass tolerances of 20
144 ppm/0.1 Da, a fixed modification of carbamidomethylation at cysteine, and variable modifications
145 of acetylation at the protein N-terminus, oxidation at methionine, deamidation at asparagine or
146 glutamine, phosphorylation at serine or threonine and phosphorylation at tyrosine were used.
147 Peptide false discovery rates (FDR) were adjusted to $\leq 1\%$ using the Mascot Percolator algorithm
148 (Käll *et al.*, 2007) and only peptides with a Mascot ion score over 13 were considered.

149 Custom scripts written in Python were implemented to parse results following data normalization
150 and quantification in Progenesis QI for proteomics. Shared peptides between proteins were
151 grouped together to satisfy the principle of parsimony and represented in Table S1 by the protein
152 accession with the highest amount of unique peptides, otherwise the largest confidence score
153 assigned by Progenesis QI for proteomics. Additionally, the script appended site localization of
154 variable modifications using an implementation of the Mascot Delta Score (Savitski *et al.*, 2011)
155 to the peptide measurements (*.csv) export from Progenesis QI for proteomics with confident site
156 localization considered a Mascot Delta score $>90\%$. Following scoring, only peptides with
157 phosphorylation at serine, threonine, or tyrosine were considered for further processing and
158 analysis.

159 *Downstream bioinformatics analysis.*

160 Missing value imputation was performed on logarithmized normalized abundances in Perseus
161 v1.6.0.0 (Cox & Mann, 2012; Tyanova *et al.*, 2016) requiring at least three of the five replicates
162 in all drug conditions and control to be nonzero to continue through the workflow. A coefficient
163 of variation (CV) cutoff was applied requiring $CV < 25\%$ in at least 2 of 4 conditions for each
164 phosphosite. For t-test analyses, replicates were grouped and the statistical tests were performed
165 with fold change threshold of ± 2 and $p \leq 0.05$ significance threshold. KEGG pathway annotation
166 (Kanehisa & Goto, 2000), Gene Ontology (GO) (Ashburner *et al.*, 2000) term annotation,

167 hierarchical clustering, and motif analysis were performed following statistical testing to glean
168 biological insight on modulated sites found in the study. For hierarchical clustering, visualization
169 was performed in Perseus v1.6.0.0. Following data normalization and missing value imputation,
170 intensity values were z-score normalized and grouped using k-means clustering with default
171 parameters. For motif analysis, sequence logo visualizations were performed using pLOGO with
172 serine or threonine residues fixed at position 0. Positions with significant residue presence are
173 depicted as amino acid letters sized above the red line (O'shea *et al.*, 2013).

174 *Carotenoid analysis.*

175 Chlamydomonas cells were collected by centrifugation (4000 g for 5 min) and resuspended in 80%
176 acetone. Samples were heat up for 5 min in a water bath at 90°C and then centrifuge at 10000g
177 10min. The supernatant evaporated under N₂, and then resuspended in 80% acetone. The
178 separation and chromatographic analysis of pigments was performed in a HPLC using a Waters
179 Spherisorb ODS2 column (4.6 x 250 mm, 5µm particle size). The chromatographic method
180 described by Baroli *et al.*, 2003 (Baroli *et al.*, 2003). Pigments were eluted at a flow rate of 1.0 mL
181 min⁻¹ with a linear gradient from 100% solvent A (acetonitrile:methanol:0.1mM Tris-HCl pH 8.0
182 [84:2:14]) to 100% solvent B (methanol:ethyl acetate [68:32]) for 20 min, followed by 7 min of
183 solvent B, then 1 min with a linear gradient from 100% solvent B to 100% solvent A, and finally
184 6 min with solvent A. The carotenoids were detected at 440 nm using a Waters 2996 photodiode-
185 array detector. The different carotenoids were identified using standards from Sigma (USA) and
186 DHI (Germany). This analysis was normalized by dry cell weight. Dry weight was determined by
187 filtering an exact volume of microalgae culture (30 mL) on pre-targeted glass-fiber filters (1µm
188 pore size). The filter was washed with a solution of ammonium formate (0.5 M) to remove salts
189 and dried at 100 °C for 24 h. The dried filters were weighed in an analytical balance and the dry
190 weight calculated by difference.

191 *SDS-PAGE and Western Blotting.*

192 Chlamydomonas cells from liquid cultures were collected by centrifugation (4000 g for 5 min),
193 washed in 50 mM Tris-HCl (pH 7.5), 10 mM NaF, 10 mM NaN₃, 10 mM p-nitrophenylphosphate,
194 10 mM sodium pyrophosphate, and 10 mM b-glycerophosphate), and resuspended in a minimal
195 volume of the same solution supplemented with Protease Inhibitor Cocktail (Sigma). Cells were
196 lysed by two cycles of slow freezing to -80 °C followed by thawing at room temperature. The

197 soluble cell extract was separated from the insoluble fraction by centrifugation (15 000 g for 20
198 min) in a microcentrifuge at 4 °C. For immunoblot analyses, total protein extracts (20 µg) were
199 subjected to 12% SDS–PAGE and then transferred to PVDF membranes (Millipore). Anti-P-
200 RPS6(Ser242) and anti-RPS6 primary antibodies were generated as described in Dobrenel et al.,
201 2016 (Dobrenel *et al.*, 2016b) and produced by Proteogenix, (France). Phospho-p70 S6 kinase
202 (Thr(P)-389) polyclonal antibody (Cell Signaling, 9205) was used as described in Xiong et al.,
203 2012 (Xiong, Yan & Sheen, Jen, 2012). Primary antibodies were diluted 1:2000 and 1:1000
204 respectively. Secondary anti-rabbit (Sigma) antibodies were diluted 1:5000 and 1:10 000,
205 respectively, in phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween-20 (Applichem)
206 and 5% (w/v) milk powder. The Luminata Crescendo Millipore immunoblotting detection system
207 (Millipore) was used to detect the proteins. Proteins were quantified with the Coomassie dye
208 binding method (BioRad).

209 **Results**

210 *Parameter selection for TORC1-specific inhibition.*

211 Previous studies in *Chlamydomonas* have shown rapamycin drug saturation ranging from 500 nM-
212 1µM (Crespo *et al.*, 2005). For this study, 500 nM rapamycin was selected and saturating doses
213 for Torin1 and AZD8055 in wild-type *Chlamydomonas* strain CC-1690 were determined using
214 serial dilutions with previously published target concentrations (Couso *et al.*, 2016). Growth
215 inhibition saturated at 500 nM for Torin1 and 700 nM for AZD8055 (Supplemental Figure 1).

216 While reports have shown phosphorylation changes as early as 2 minutes after rapamycin
217 treatment (Rigbolt *et al.*, 2014), a 15-minute time point was chosen based on the high number of
218 changes seen in mammalian cell lines at this time point (Demirkan *et al.*, 2011; Harder *et al.*, 2014;
219 Rigbolt *et al.*, 2014) and to ensure reproducibility in treatment and harvesting across 20 samples
220 (control, AZD8055-, Torin1-, and rapamycin-treated with n=5) from the early logarithmic phase
221 of growth. Growth for each replicate was staggered, and to limit batch-effects replicates were
222 harvested in sets, each containing a control sample and the three different drug-tested samples
223 (Figure 1) prior to downstream processing.

224 Prior rapamycin phosphoproteomic experiments in mammalian studies have shown that
225 phosphopeptide ratios in general were not affected by normalization to protein levels at a 15 min

226 time point (Harder *et al.*, 2014). To confirm this in *Chlamydomonas reinhardtii*, a whole-cell
227 proteomics experiment (n=4) was performed after 15 min of rapamycin inhibition. These results
228 showed that protein abundance levels in general are not affected with only 18 of the 1,539 proteins
229 quantified significantly changing (Supplemental Table S4) with no significant differences in
230 protein abundances between control and treatment (Supplemental Figure 2). While 4 of the 18
231 proteins changing at the protein level were identified in the phosphoproteomics study detailed
232 below, they were not detected as phospho-modulated following chemical inhibition and thus not
233 proteins of interest in this study. Thus, we have confidence that the statistically significant
234 phosphorylation sites detected are from changes in the phosphorylation status and not an artefact
235 of protein expression or turnover.

236 *Quantitative coverage of the TOR-inhibited phosphoproteome.*

237 Label-free quantitative phosphoproteomics was used to compare normalized abundance values of
238 control samples (n=5) versus samples treated with each of the chemical inhibitors (n=5) using an
239 area under the curve (AUC) MS1 intensity-based quantitation method. For this approach, the
240 change in chromatographic peak area between control and chemically-inhibited replicates for each
241 phosphopeptide was the basis for determining relative phosphopeptide abundance. Tip-based TiO₂
242 phosphopeptide enrichment that previously showed high reproducibility between samples (Werth
243 *et al.*, 2017) was used for sample preparation. As part of the LFQ pipeline, quantitative data was
244 filtered for only peptides containing a phosphorylation site on Ser, Thr, or Tyr after peak picking
245 and peptide sequence determination. At least 3 of the 5 replicates for each condition were required
246 to have nonzero abundances to remain in the final dataset presented in Table S1 and missing value
247 imputation was performed on log-transformed normalized abundances (Cox & Mann, 2012;
248 Tyanova *et al.*, 2016). Highly variable sites remaining in the dataset were then removed by filtering
249 out those with a coefficient of variation of >25% in >2 experimental conditions. The resulting
250 dataset contained 2,547 unique phosphosites from 1,432 different proteins (Table S1) in untreated
251 control samples. To determine sites of interest following chemical inhibition with Torin1,
252 AZD8055, or rapamycin, two sample Student's T-tests were performed between samples from
253 each chemical inhibitor compared and control samples. From this, 258 phosphosites from 219
254 phosphopeptides showed at least a two-fold change and a p-value ≤ 0.05 (Figure 2a, Table S2).
255 High confidence phosphorylation site assignments (90% site-localization based on Mascot Delta

256 scoring(Savitski *et al.*, 2011)) were achieved for 48% of the dataset (1,123 of the 2,363
257 phosphopeptides) listed in Table S1. AZD8055 treatment resulted in 97 phosphopeptides
258 modulated in the wild-type strain (Figure 2a). A matched control experiment using an AZD-
259 insensitive strain which grow similar to wild-type (Supplemental Figure 3) showed only 13 low
260 abundance phosphosites differentially changing (Table S3, Figure 2b). Of the 13, no overlap was
261 found with the 258 modulated phosphosites in the main dataset.

262 Torin1 treatment caused the largest number of significant changes with 103 up- and 57 down-
263 modulated phosphosites. AZD8055 treatment caused 75 up- and 19 down-modulated
264 phosphosites, while rapamycin treatment caused 40 up- and 35 down-modulated phosphosites.
265 Overlap analysis of the differential sites for each drug revealed 88% (57/66) of all the down-
266 modulated sites were in the Torin1 subset, while 42% (24/57) of the Torin1 down-modulated sites
267 were not detected with AZD or rapamycin. Up-regulated sites were also compared for each
268 condition and to determine if the conditions had significant overlap between down- and up-
269 modulated sites, a hypergeometric test was performed with p-values of 3.76×10^{-25} and 2.87×10^{-34} ,
270 respectively, showing significant overlap.

271 *Cluster analysis and phosphosite motif identification.*

272 Kinase specificity can be dictated by amino acid residues immediately surrounding
273 phosphorylation sites on substrates (Chou & Schwartz, 2011). Mammalian TOR has been shown
274 to mainly (but not exclusively) phosphorylate (S/T)P motifs and motifs with hydrophobic residues
275 surrounding the phosphorylation site making it a relatively promiscuous kinase whose substrate
276 choices may also be influenced by additional interactions outside the phosphosite region
277 (Robitaille *et al.*, 2013). Hierarchical clustering of *Chlamydomonas* modulated phosphosites
278 generated 2 distinct clusters (Figure 3a,b), and motif analysis (O'shea *et al.*, 2013) was performed
279 on decreasing (cluster 1) and increasing (cluster 2) clusters. Cluster 1 phosphosites, which
280 contained 94% of sites that significantly decrease in phosphorylation upon TOR inhibition, had
281 significant enrichment for a proline in the +1 position and arginine in the -3 position with respect
282 to the phosphorylation site (position 0) that showed strong enrichment for serine over threonine
283 (Figure 3c). Cluster 2 phosphosites also had significant enrichment for a proline in the +1 position
284 and arginine in the -3 position in addition to enrichment for an aspartic acid at the +3 position.
285 Thus, CrTOR may have a preference for phosphorylation of (S/T)P motifs on substrates, similar

286 to mTOR(Robitaille *et al.*, 2013) and other diverse proline-directed kinases including cyclin-
287 dependent protein kinases (CDKs) and mitogen-activated protein kinases (MAPKs) (Lu *et al.*,
288 2002). Additionally, a phosphoproteomic study using mammalian cell line MCF7 identified the
289 RXXS/TP motif identified in clusters 1 and 2 as a rapamycin-sensitive motif (Rigbolt *et al.*, 2014).
290 Other studies have also found RXRXXS/T and RXXS/T motifs (Demirkan *et al.*, 2011; Harder *et*
291 *al.*, 2014) enriched among rapamycin-sensitive phosphosites that are recognized by mTOR-
292 regulated kinases Akt, S6K1 and SGK1 (Hsu *et al.*, 2011). Cluster 2 additionally has an acidic
293 motif also found in casein kinase- II substrates (Lv *et al.*, 2014).

294 *Phosphosites in TORC1 complex proteins.*

295 Numerous phosphosites in mammalian homologs of TORC1 complex proteins are regulated by
296 the TOR pathway and/or are phosphorylated autocatalytically (Foster *et al.*, 2010). This includes
297 sites on Raptor and mTOR homologs. Therefore, phosphosites found on CrTORC1 complex
298 proteins could be affected by TOR inhibition. TORC1 complex proteins conserved in
299 Chlamydomonas include TOR (Cre09.g400553.t1.1), Raptor (Cre08.g371957.t1.1), and LST8
300 (Cre17.g713900.t1.2) (Merchant *et al.*, 2007; Diaz-Troya *et al.*, 2008; Perez-Perez *et al.*, 2010;
301 Couso *et al.*, 2016). While there is a known LST8 homolog in Chlamydomonas, it is not known to
302 be phosphorylated (Wang *et al.*, 2014). Phosphosites on Raptor (Ser782/783:NL) (Not
303 Localized:NL) and TOR (Ser2598) were detected in this study, however no statistically significant
304 modulation in their abundance was detected. BLASTP alignment of human Raptor (Uniprot
305 Q8N122) with CrRaptor revealed high sequence overlap on the N-terminal region of the protein
306 (residues 9-627 with 57% identity), however known TORC1-sensitive phosphosites in the human
307 Raptor homolog (i.e. Ser719, Ser721, Ser722, Ser859, and Ser863 (Carrière *et al.*, 2008; Foster *et*
308 *al.*, 2010)) were not conserved in CrRaptor. Similarly, human mTOR (Uniprot P42345)
309 phosphosites Ser2159/Thr2164 that are within the kinase domain promoting mTORC1-associated
310 mTOR Ser2481 autophosphorylation (Ekim *et al.*, 2011) are not conserved in CrTOR. The limited
311 sequence conservation among CrTORC1 phosphosites with mammalian TOR phosphosites
312 precludes any predictions about functions of CrTORC1 protein phosphorylation. Other
313 phosphosites on CrTORC1 complex proteins that were detected in previous work on the global
314 phosphoproteome in Chlamydomonas (Wang *et al.*, 2014) might be significant for regulation but
315 they were not observed in our data. Future experiments with additional fractionation to increase

316 the dynamic range of quantitative coverage could allow for deeper coverage and more
317 comprehensive detection of phosphosites.

318 **Discussion**

319 *Sites modulated by TORC1 inhibition – known and putative substrates.*

320 In animal cells TORC1-inhibition blocks phosphorylation of multiple substrates including S6
321 kinases and eukaryotic translation initiation factors, leading to a reduction in translation initiation
322 rates for a subset of mRNAs (Jefferies *et al.*, 1994; Terada *et al.*, 1994; Wang & Proud, 2009).
323 Phosphorylation of Ser371 and Thr389 in human p70S6K1 (Uniprot P23443-2) are reduced by
324 treatment of cells with TOR inhibitors (Dennis *et al.*, 1996; Burnett *et al.*, 1998). While we
325 identified one potential site (site was not localized) (Thr771/Ser773/Thr777:NL) on a
326 Chlamydomonas homolog of ribosomal protein S6 kinase (S6K; Cre13.g579200.t1.2), its
327 phosphorylation state was not significantly altered by TOR inhibitors (Table 1). No coverage was
328 obtained on predicted conserved sites Ser915 and Thr932, which align to human p70S6K1 Ser371
329 and Thr389, respectively, although these sites have been detected previously in Chlamydomonas
330 (Wang *et al.*, 2014). Moreover, while commercial anti-phospho S6K antibodies have been shown
331 to detect phospho-S6K in plants (Xiong, Yan & Sheen, Jen, 2012; Ahn *et al.*, 2014) they have not
332 detected a signal in Chlamydomonas in our hands (Supplemental Figure 4) and in another study
333 (Couso *et al.*, 2016), thus limiting our ability to independently validate Chlamydomonas TOR
334 substrate phosphopeptides. On the other hand, Chlamydomonas ribosomal protein S6 (RPS6,
335 Cre09.g400650.t1.2), a predicted target of S6K, showed a 2.1-fold decrease in phosphorylation on
336 Thr127 following Torin1 treatment (Figure 5, Table 1). While this site is potentially TORC1-
337 regulated, antibodies specific for this phosphosite needed for validation are not available. In
338 Arabidopsis, a phosphosite on the C-terminal extremity peptide of RPS6, Ser240, had decreased
339 phosphorylation following TOR inactivation (Dobrenel *et al.*, 2016b). While this exact site is not
340 conserved in Chlamydomonas, the phosphoserine next to it, Ser241 in Arabidopsis (aligning to
341 Ser242 in Chlamydomonas) has been detected in prior work (Wang *et al.*, 2014); however it was
342 not detected in this study (Figure 4a). To determine if Ser242 in Chlamydomonas is TORC1-
343 regulated, a western blot of proteins fractionated from wild-type cells under different drug
344 treatments for 0, 5, 15, 30, and 60 min was performed with antibodies raised for phosphorylated
345 and non-phosphorylated Ser242 (Figure 4b), the latter used as a control for monitoring protein

346 level. Interestingly, this site does not seem to change drastically with Torin1, AZD8055, or
347 rapamycin treatment contrary to results on the C-terminal phosphosite in Arabidopsis.

348 *Sites modulated by TORC1 inhibition – known TOR pathway association.*

349 Of the 258 phosphosites detected as significantly modulated in this study, 10 are in homologs of
350 proteins associated with the TOR signaling pathway (Figure 5, Table 1). In addition to four sites
351 of decreasing phosphorylation, six proteins related to the TOR pathway had an increase in protein
352 phosphorylation following chemical inhibition. While initially an unexpected observation, similar
353 increases were previously reported for some phosphosites in a phosphoproteomic study of TOR
354 inhibition in mouse liver (Demirkan *et al.*, 2011). In our study, sites with increasing
355 phosphorylation after TOR inhibition include elongation factor 2 (EEF2, Cre12.g516200.t1.2)
356 whose animal homologs showed reduced activity upon phosphorylation. In human cells,
357 phosphorylation of EEF2 Thr57 by elongation factor 2 kinase (EEF2K, Cre17.g721850.t1.2)
358 inactivates EEF2 activity, an essential factor for protein synthesis (Hizli *et al.*, 2013). This site is
359 conserved in Chlamydomonas EEF2 (Thr57/Thr59:NL) where we detect a 4.75-fold increase in
360 phosphorylation with AZD8055 treatment with a predicted effect of reduced translation initiation
361 rates. From these data we predict that CrTOR signaling may inhibit EEF2 kinase activity, and that
362 this inhibition is relieved in the presence of TOR inhibitors.

363 LA RNA-binding protein (LARP1, Cre10.g441200.t1.2) had two phosphosites that both
364 underwent large decreases in phosphorylation upon treatment with the three chemical inhibitors.
365 Ser817 was decreased 0.06_{AZD8055}, 0.05_{Torin1}, and 0.13_{RAP} and Ser 737/738:NL was decreased
366 0.08_{AZD8055} and 0.01_{Torin 1} but no change in rapamycin (0.99_{RAP}) (Figure 5). In mammals, LARP1
367 phosphorylation also requires mTORC1 (Hsu *et al.*, 2011; Yu *et al.*, 2011; Kang *et al.*, 2013) with
368 studies in human cell lines establishing LARP1 as a target of mTORC1 and S6K with non-
369 phosphorylated LARP1 interacting with both 5' and 3' UTRs of RP mRNAs and inhibiting their
370 translation (Hong *et al.*, 2017). Additional reports have shown LARP1 as a direct substrate of
371 mTORC1 in mammalian cells with mTORC1 controlling Terminal Oligopyrimidine (TOP)
372 mRNA translation via LARP1 (Fonseca *et al.*, 2015; Hong *et al.*, 2017). The dramatic modulation
373 of LARP1 phosphorylation detected in our study indicates that LARP1 may have a parallel role in
374 Chlamydomonas. The human LARP1 phosphosites are not conserved with those we found in
375 Chlamydomonas. However, based on the NCBI conserved domain searching (Marchler-Bauer &

376 Bryant, 2004), the DM15 domain required for the interaction of LARP1 with mTORC1 in human
377 cell lines is conserved in *Chlamydomonas* LARP1, and the phospho-Ser817 detected in our study
378 is adjacent to the DM15 domain (877-915) in *Chlamydomonas*, a region in mammalian
379 LARP1 shown to be required for interaction with mTORC1 (Hong *et al.*, 2017).

380 *Additional proteins with phosphosites altered by TORC1 inhibition*

381 The majority of differential phosphosites we identified were not previously linked to TOR
382 signaling, including in *Chlamydomonas*. These include sites on a translation-related protein
383 (Cre17.g696250.t1.1) and RNA-binding proteins (Cre10.g441200.t1.2, Cre10.g466450.t1.1,
384 Cre16.g659150.t1.1, Cre16.g662702.t1.1 Cre17.g729150.t1.2). One of the most down-modulated
385 proteins annotated as CTC-interacting domain 4 (CID4, Cre01.g063997.t1.1), has been shown to
386 have an important function in regulation of translation and mRNA stability in eukaryotes (Bravo
387 *et al.*, 2005; Jiménez-López *et al.*, 2015). CID4 had 2 sites, Ser441 (FC=0.2_{AZD8055}, FC=0.14_{TORIN1})
388 and Ser439/Ser441/Ser446:NL (FC=0.03_{AZD8055}, FC=0.05_{TORIN1}) with a large decrease in
389 phosphorylation upon inhibitor treatment. While little is known about the relationship between this
390 protein and TORC1 signaling, the CTC domain, more recently referred to as the MLLE domain
391 (Jiménez-López & Guzmán, 2014), is also found in evolutionarily conserved Poly (A)-binding
392 proteins (PABPs). The large decrease in CID4 phosphorylation seen upon inhibition of the
393 CrTORC1 pathway in our study implies a potential role for TORC1 mediated control of
394 translation, similar to other well-known TOR substrates.

395 Another differential phosphosite of interest following TORC1 inhibition that was not previously
396 linked to TOR regulation is a site on lycopene beta/epsilon cyclase protein (Cre04.g221550.t1.2--
397 Thr800/Ser802:NL). This phosphosite is significantly increased upon Torin1 treatment (FC=4.02)
398 and the total protein level remained constant upon rapamycin treatment (Supplementary Table S4,
399 FC=0.88). Lycopene beta/epsilon cyclases are required for carotenoid biosynthesis, carrying out
400 cyclation of lycopene to yield α - and β - carotenes (Cunningham *et al.*, 1996; Cunningham & Gantt,
401 2001; Cordero *et al.*, 2010) which have been shown to be high-value compounds participating in
402 light harvesting and in the protection of the photosynthetic apparatus against photo-oxidation
403 damage (Frank & Cogdell, 1996; Cunningham Jr & Gantt, 1998). Recently in rice, carotenoid
404 content was shown to be significantly lower in an *s6k1* mutant compared to wild-type (Sun *et al.*,
405 2016) revealing a potential connection between the TOR pathway and carotenoid production. To

406 further investigate the effect of TORC1 inhibition on carotenoid biosynthesis in *Chlamydomonas*
407 based on our phosphoproteomic finding, carotenoid levels in AZD-, Torin1- and rapamycin-treated
408 cells were assessed after eight hours of treatment with three biological and two technical replicates
409 (Figure 6, Table 2). After eight hours of treatment, there was a significant increase in various
410 carotenoids measured in TOR-inhibited samples including β -carotene, which is directly
411 downstream of cyclase activity (Figure 6, Table 2). While the effects on carotenoid biosynthesis
412 and secondary metabolism following TORC1 inhibition required eight hours to become detectable,
413 this is the first evidence that carotenoid production is modulated by TOR signaling in algae.
414 Additionally, altered cyclase protein levels are not likely responsible for this finding since previous
415 studies showed no change in lycopene beta/epsilon cyclase protein level after up to 24 hours of
416 nitrogen stress (Cunningham Jr & Gantt, 1998; Valledor *et al.*, 2014), a condition that is
417 metabolically similar to TOR inhibition (Perez-Perez *et al.*, 2010; Roustan *et al.*, 2017).

418 Numerous phosphosites from proteins without Phytozome database descriptions were also found
419 to be down-regulated upon CrTORC1 inhibition, including some sites with large decreases (>five-
420 fold). For all unannotated proteins, we searched for pfam, Panther, KOG, KEGG, KO, and GO
421 pathway terms and domain conservation using Phytozome and NCBI annotations (Table S4).
422 Numerous proteins had conserved domains including structural maintenance of chromosomes
423 (Accession: cl25732), autophagy protein (Accession: cl27196), transmembrane proteins
424 (Accession: cl24526), and small acidic protein (Accession: pfam15477). While the large changes
425 upon chemical inhibition are potentially interesting, especially the five proteins containing sites
426 with at least a five-fold decrease in phosphorylation (Cre03.g152150.t1.2, Cre06.g263250.t1.1,
427 Cre11.g469150.t1.2, Cre05.g236650.t1.1, Cre13.g582800.t1.2), future targeted work would be
428 required to infer biological significance to this observation. To aid in this, the fifty-eight modulated
429 sites without Phytozome database annotation were also homology searched for best BLAST hit
430 IDs in *Volvox*, *Gonium*, and *Arabidopsis* to find homologs among green lineage (Table S5) and
431 Table S2 displays all of the experimentally derived sites modulated by AZD8055, Torin1, and/or
432 rapamycin and will serve as a guide in follow-up studies.

433 In summary, we obtained a candidate list of phosphosites modulated following TORC1
434 inhibition. We achieved extensive coverage of the TOR-modulated phosphoproteome in
435 *Chlamydomonas* using a quantitative label-free approach. Our approach was validated by the

436 overlap of phosphosites altered using different TOR inhibitors and by our identification of
437 Chlamydomonas homologs of TOR signaling-related proteins such as RPS6 and LARP1 that had
438 decreased phosphorylation upon TORC1 inhibition. Follow-up experiments guided by our
439 phosphoproteomic findings in lycopene beta/epsilon cyclase showed that carotenoid levels are
440 affected by TORC1 inhibition, the first evidence that carotenoid production is under TOR control
441 in algae. Conserved TOR substrate motifs were also identified such as RXXS/TP and RXXS/TP.
442 Our study provides a new resource for investigating the phosphorylation networks governed by
443 the TOR kinase pathway in Chlamydomonas.

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448 E.G.W., L.M.H., I.C.L., J.G.U., J.L.C. contributed to planning and experimental design. E.G.W.,
449 I.C.L., and Z.P. performed experiments. E.G.W., E.W.M. performed data analysis. E.G.W.,
450 L.M.H., J.G.U wrote the manuscript.

451

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672

673

674 Tables

675 Table 1: TOR targets identified with fold change values for drug condition versus control.

Accession	Common Name	Sites	Fold-change		
			AZD8055	Torin1	Rapamycin
CrTORC1 proteins					
Cre09.g400553.t1.1	TOR	S2598	0.99	0.90	1.12
Cre08.g371957.t1.1	RAPTOR	S782/S783:NL	1.56	1.94	1.54
homologs of known substrates					
Cre13.g579200.t1.2	RPS6KB	T771/S773/T777:NL	1.17	1.35	1.05
Cre09.g400650.t1.2	RPS6	T127	0.90	0.48**	0.61*
homologs of TOR pathway-associated proteins					
Cre10.g441200.t1.2	LARP1	T668/S670:NL	1.50	1.91*	1.96*
		S737/738:NL	0.08**	0.01**	0.99
		T809/S810:NL	0.41	0.46	0.81
		S817	0.06**	0.05**	0.13**
Cre17.g721850.t1.2	EEF2K	S306	0.75	0.42**	0.58
		S589/S591:NL	1.13	1.24	1.20
		S853/S857	2.00*	2.73**	1.69
Cre12.g516200.t1.2	EEF2	T57/T59:NL	4.75*	1.88	2.76
Cre12.g511850.t1.2	GSK3B	S322	1.27	1.15	1.25*
Cre09.g391245.t1.1	ATG1	T802/S803:NL	1.80	1.65	1.53
Cre06.g251050.t1.1	PRKAA	S699/S702:NL	0.64	0.29	0.97
Cre10.g457500.t1.1	PRKAB	S25/S29:NL	1.67*	1.75	2.27**
Cre02.g100300.t1.1	PI-3K/PI-4-like	T149/S150:NL	1.11	0.82	0.92
Cre05.g245550.t1.1	PI3KA	S794	1.04	0.91	1.51
Cre06.g304650.t1.1	PI3KB2	S403	1.25	1.39	0.88
Cre03.g192000.t1.2	SEH1	T478/S479/S482:NL	0.94	1.19	1.06
		S337	4.22	2.64	1.43
Cre02.g076900.t1.1	PRKG1	S71	2.20	1.54	1.59**
		S71/S78:NL	1.44	1.76*	1.41
		S126, S128	1.84	1.23	0.82
		T857/T859:NL	1.12	0.97	0.98
		T857/T859:NL	0.89	0.97	0.92
		T857/T859/T863:NL	1.45	1.17	1.22*
		S378	1.25	0.79	0.94
Cre10.g461050.t1.2	ATP synthase A	S378	1.25	0.79	0.94
Cre02.g076350.t1.2	ATP6B, ATPase	S7/S8:NL	2.24	1.12	2.09**
Cre11.g468550.t1.2	ATP synthase G2	S7	2.52*	1.62*	2.33
		S77	1.60	1.24	1.33

*p-value ≤ 0.05 **p-value ≤ 0.01

Up- Down-

676

677 Fold change values shaded red indicate a statistically significant increase in phosphopeptide
 678 abundance for specified drug treatment versus control. Fold change values shaded blue indicate a
 679 statistically significant decrease in phosphopeptide abundance for specified drug treatment versus
 680 control. Level of p-value statistical significance is denoted by p-value ≤ 0.05 (*) and ≤ 0.01 (**)

681 Table 2: Carotenoid content in WT Chlamydomonas after 8 hours of treatment with Rapamycin,
682 Torin1, or AZD8055 compared to control.

683

Carotenoids Content (mg g^{-1} DW)

	Control	500nM Rap	500nM Torin	700nM Azd
Neoxanthin	0.64±0.01	1.12±0.01*	1.25±0.04*	1.65±0.00*
Violaxanthin	0.50±0.00	0.57±0.00	0.93±0.07*	1.02±0.00*
Anteraxanthin	0.04±0.00	0.12±0.00*	0.16±0.01*	0.11±0.00*
Lutein	1.60±0.03	2.56±0.02*	3.42±0.14*	3.29±0.00*
B-carotene	1.82±0.03	1.80±0.03	2.09±0.02*	3.02±0.02*

684 Figure Legends:

685 Figure 1. Drug treatment and cell harvesting workflow in *Chlamydomonas* cells. Replicate “n”
686 (1-5) of each drug condition and control were harvested together prior to downstream processing.
687 To minimize inter-condition batch effects, “n” replicate of each condition was harvested together
688 and frozen until protein extraction.

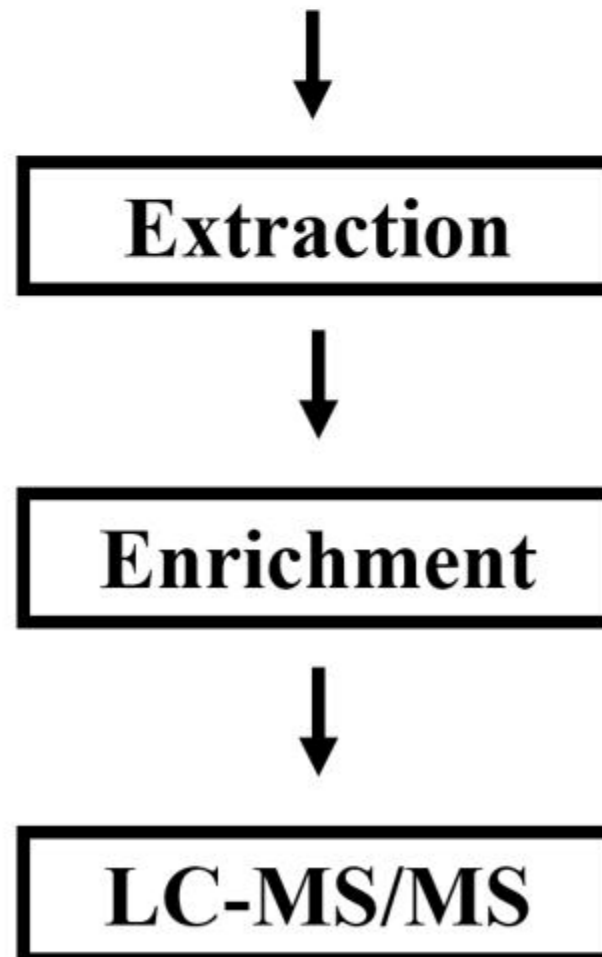
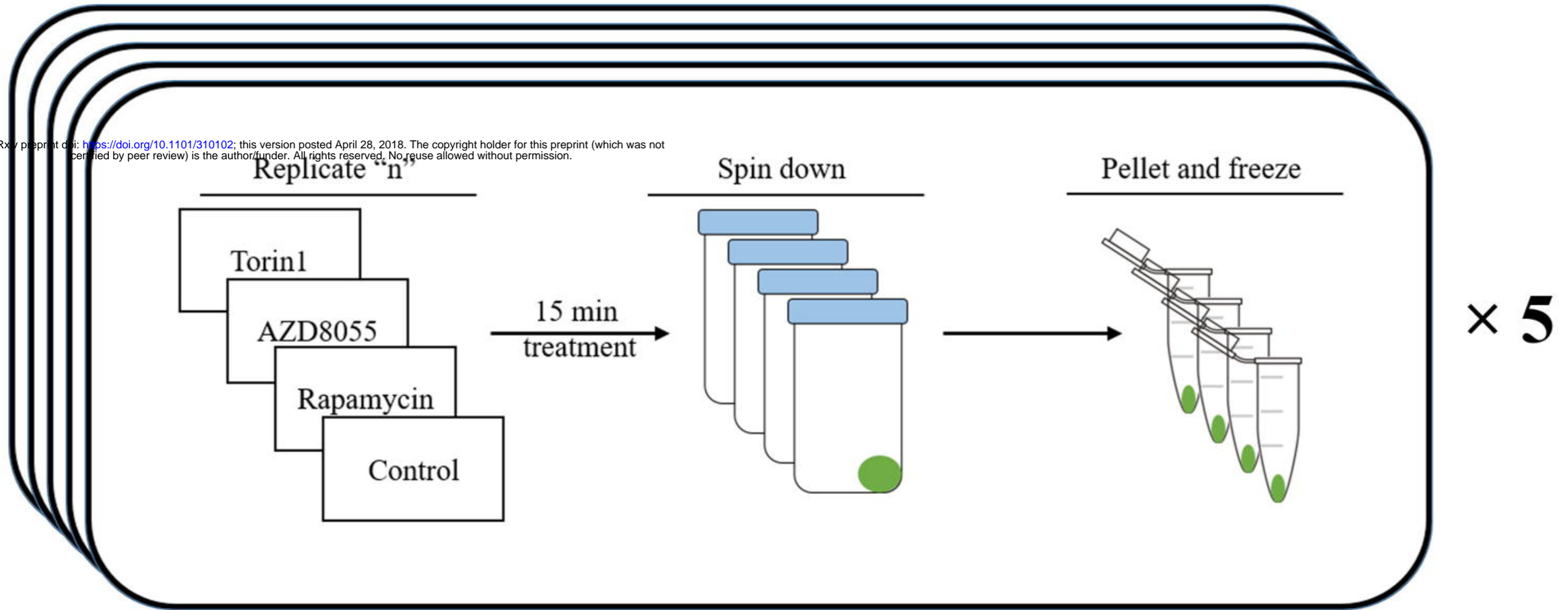
689 Figure 2: Sites modulated by TOR inhibition. Results of differential analysis between each
690 chemical inhibitor drug treatment compared to control for both wild-type (a) and AZD-
691 insensitive (b) *Chlamydomonas* strains. For comparison of overlap between the drug conditions
692 in the WT dataset, a Pearson’s correlation was performed comparing all condition types. From
693 this, the highest correlation among conditions was between AZD8055 and Torin1 at 0.986 and
694 the lowest 3 were all drug inhibitor vs. controls.

695 Figure 3. Hierarchical clustering of differentially changing sites into 2 clusters (a). Visualization
696 was performed in Perseus v1.6.0.0. Following data normalization and missing value imputation,
697 intensity values were z-score normalized and grouped using k-means clustering with default
698 parameters. Overall trends in site intensity were graphed and colored based on intensity (b). For
699 each of the two clusters, motif analysis was performed (c). Sequence logo visualizations were
700 performed using pLOGO with serine or threonine residues fixed at position 0. Positions with
701 significant residue presence are depicted as amino acid letters sized above the red line. For
702 cluster 1, there was significant enrichment for a proline in the +1 position and arginine in the -3
703 position, RXXS/TP. For cluster 2, there was again significant enrichment for a proline in the +1
704 position and arginine in the -3 position in addition to an aspartic acid in the +3 position,
705 RXXS/TPXD.

706 Figure 4 Comparison of RPS6 protein sequence between *Arabidopsis* and *Chlamydomonas* (a). a
707 western blot in wild-type under different drug treatments for 0, 5, 15, 30, and 60 min with
708 antibodies raised for Ser242 (b).

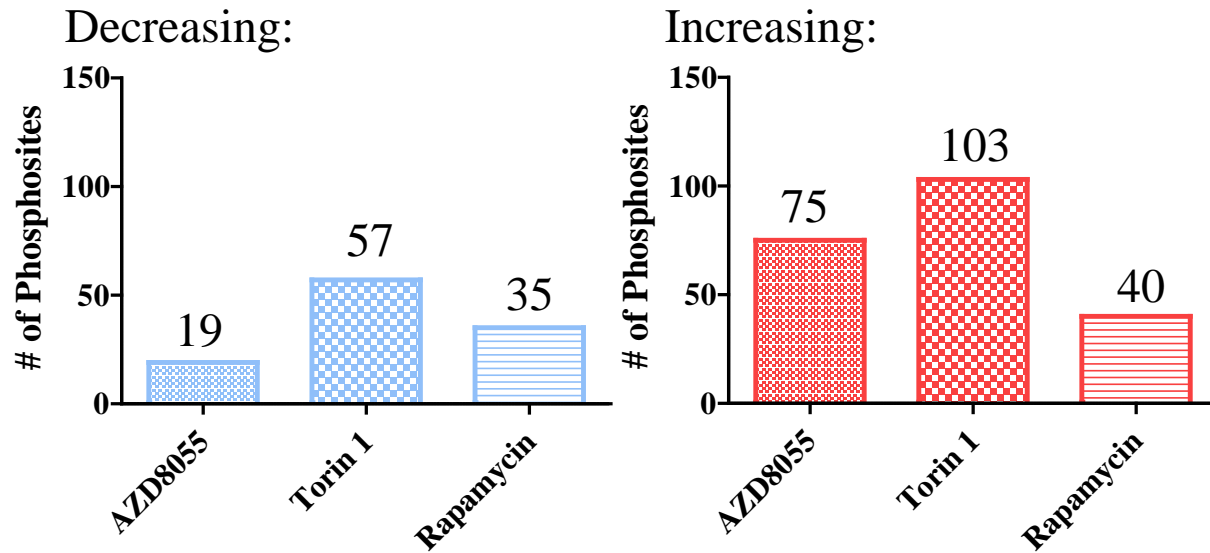
709 Figure 5: Bar charts of 10 modulated phosphosites on TOR pathway-associated proteins based
710 on homology. Level of p-value statistical significance is denoted by p-value ≤ 0.05 (*) and \leq
711 0.01 (**)

712 Figure 6: Bar chart of carotenoid content in WT Chlamydomonas after 8 hours of treatment with
713 Rapamycin, Torin1, or AZD8055 compared to control



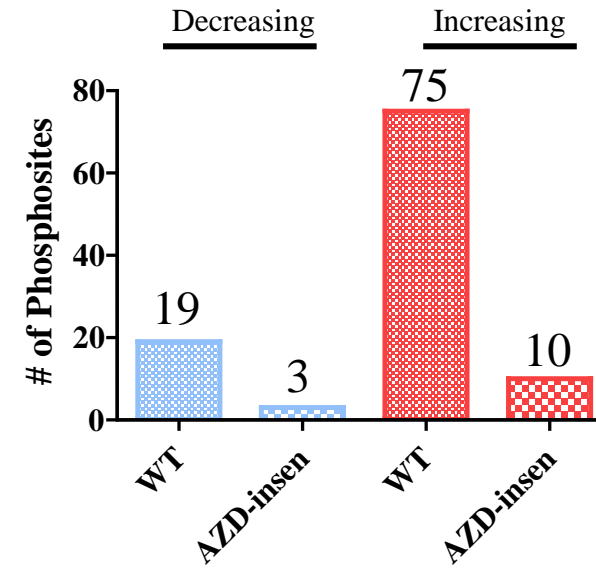
a.

Wild Type
258 phosphosites differentially changing

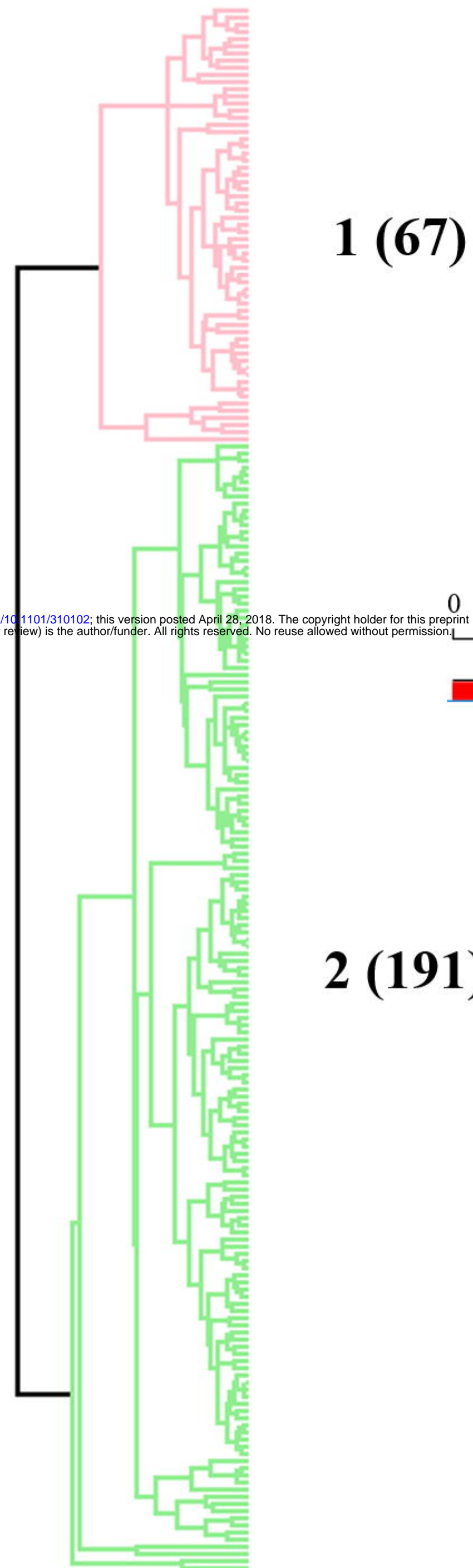


b.

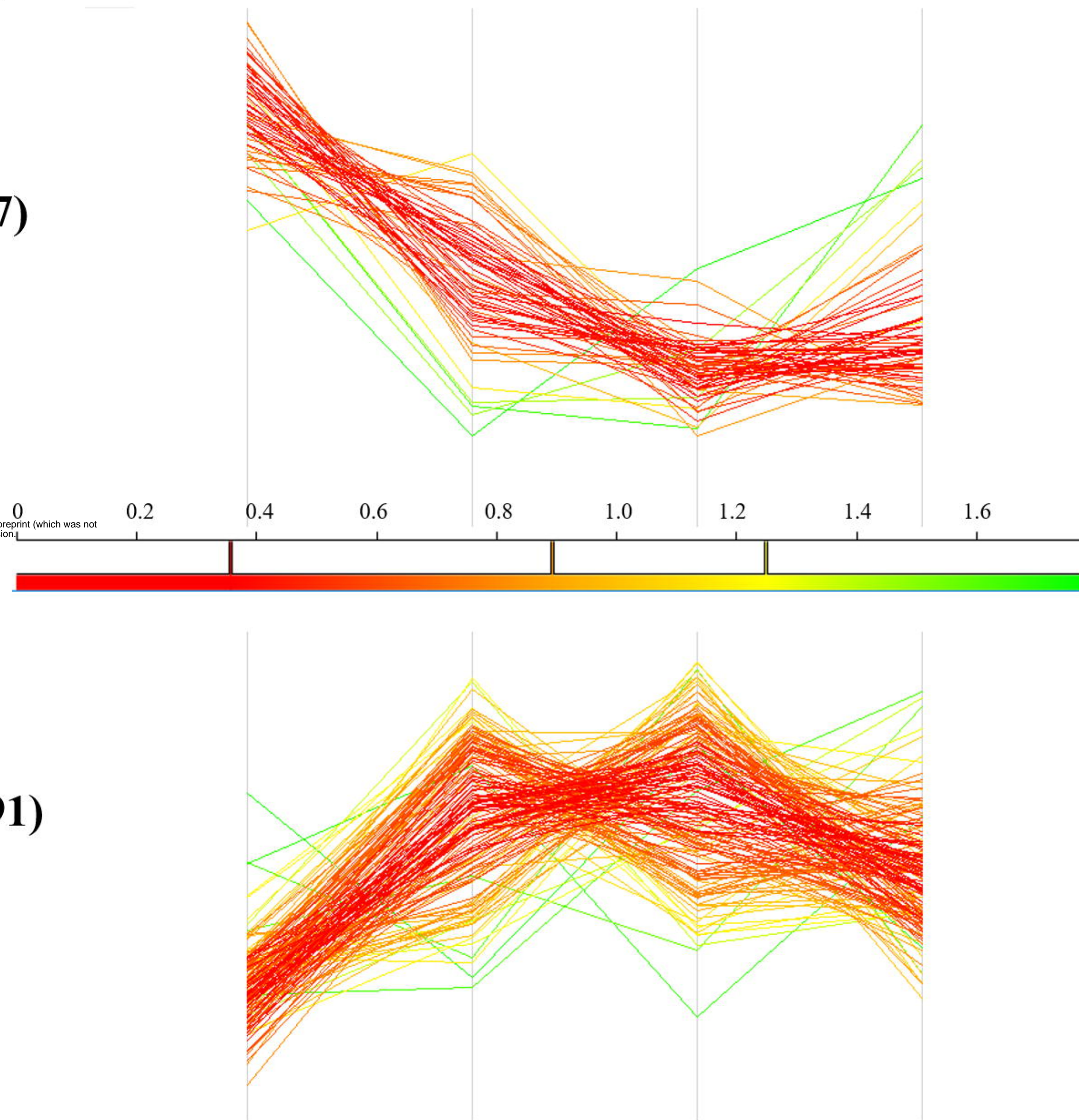
AZD-insensitive
13 phosphosites differentially changing



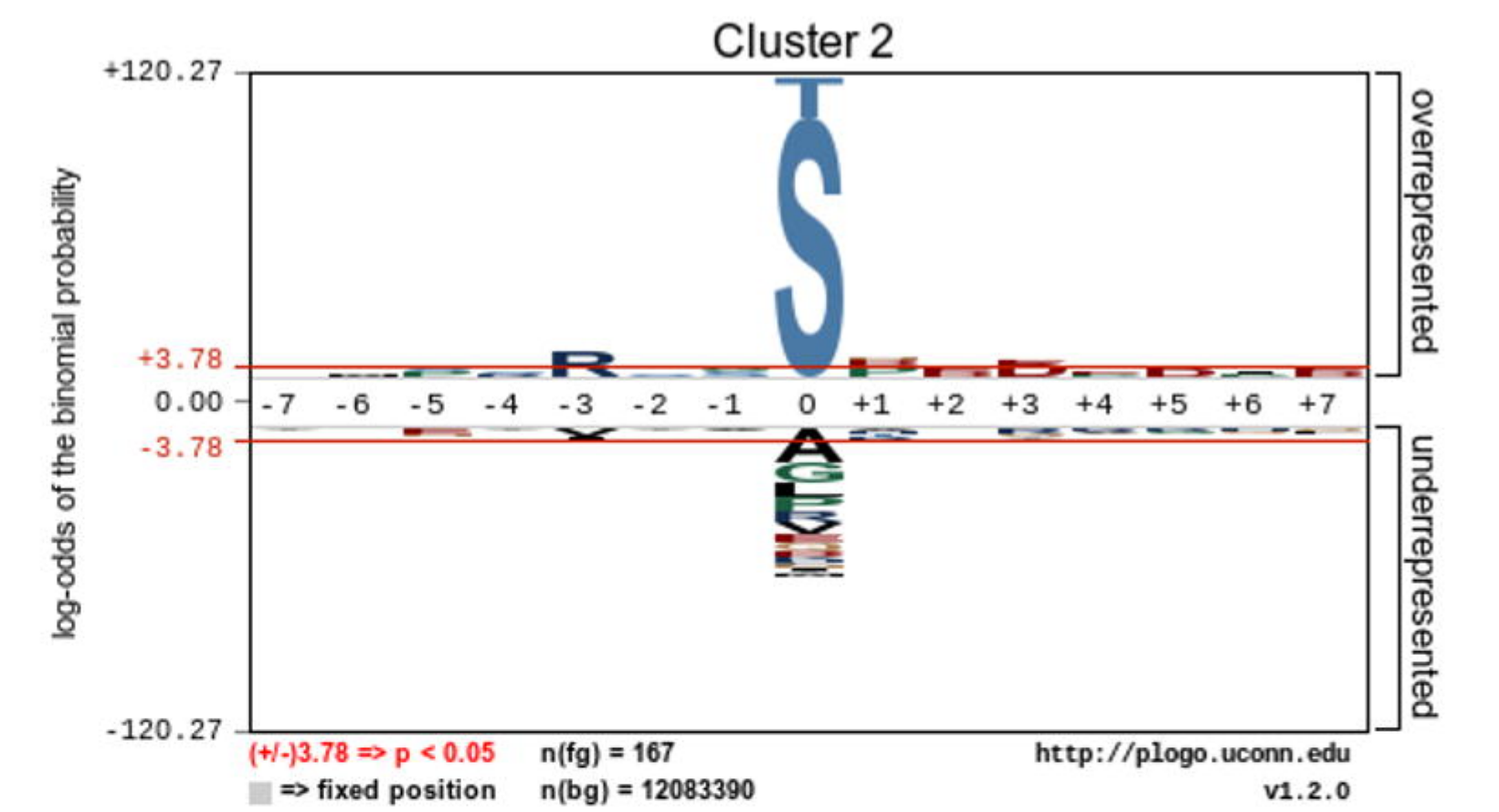
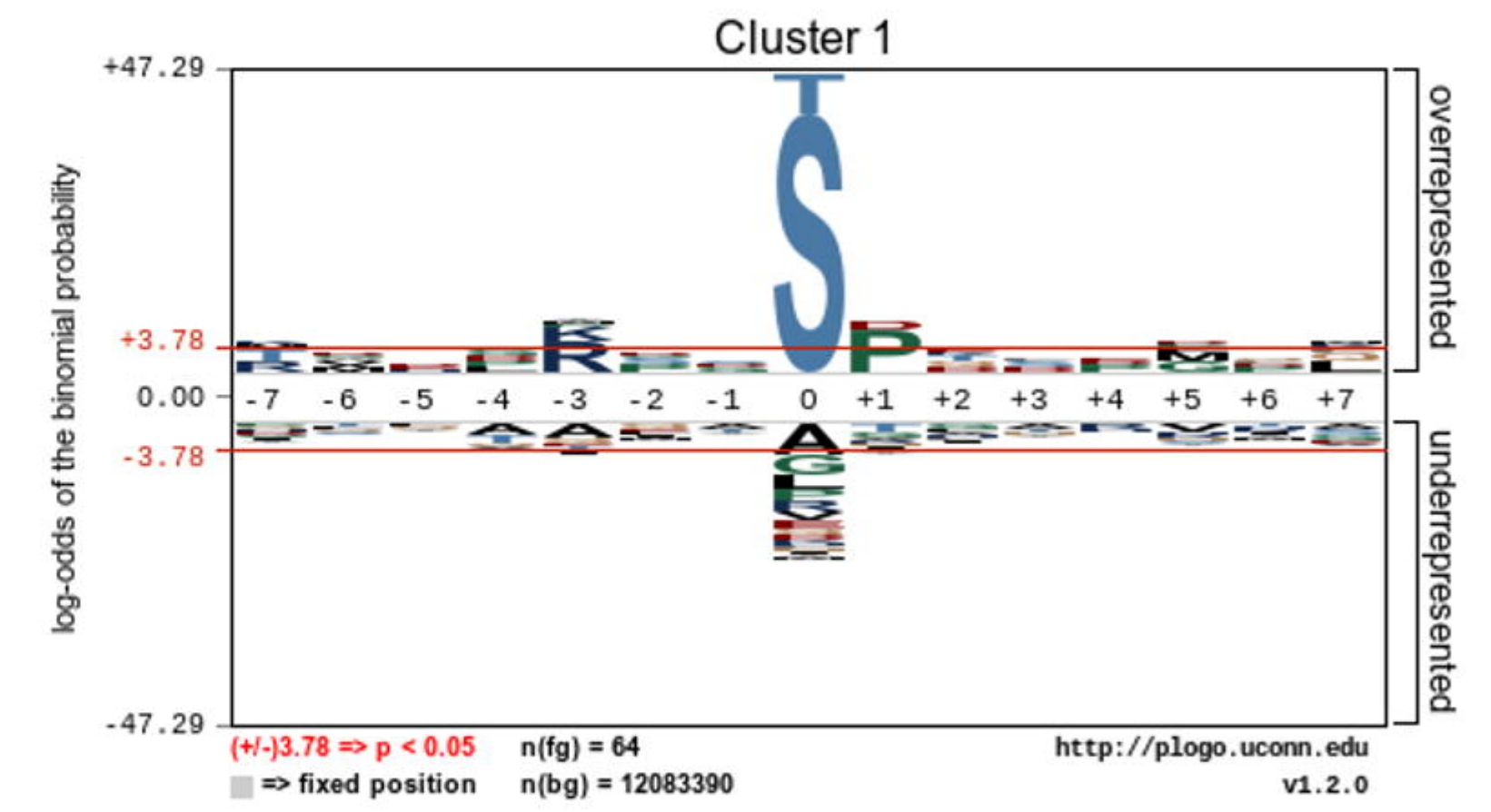
a. Cluster (# of phosphosites)



b. Control AZD8055 Torin 1 Rapamycin



c.

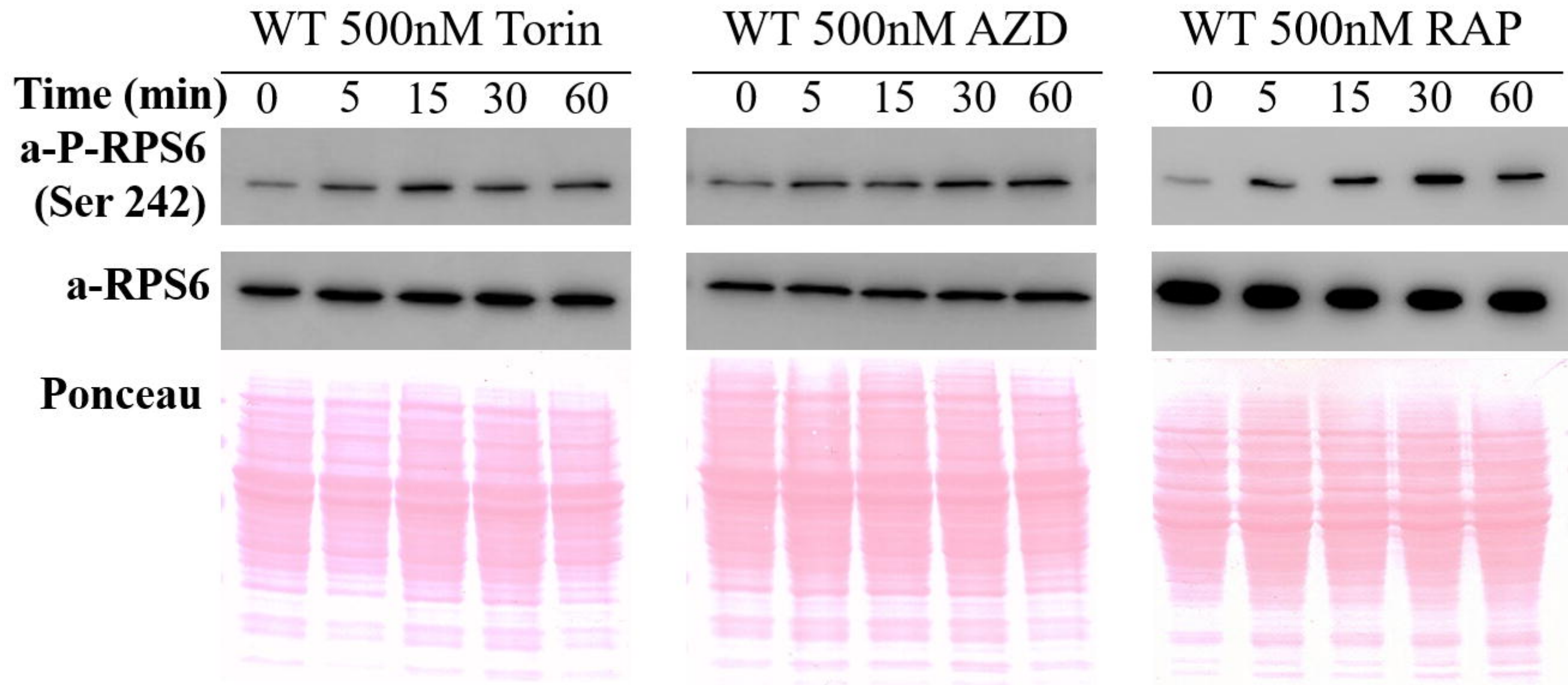


a.

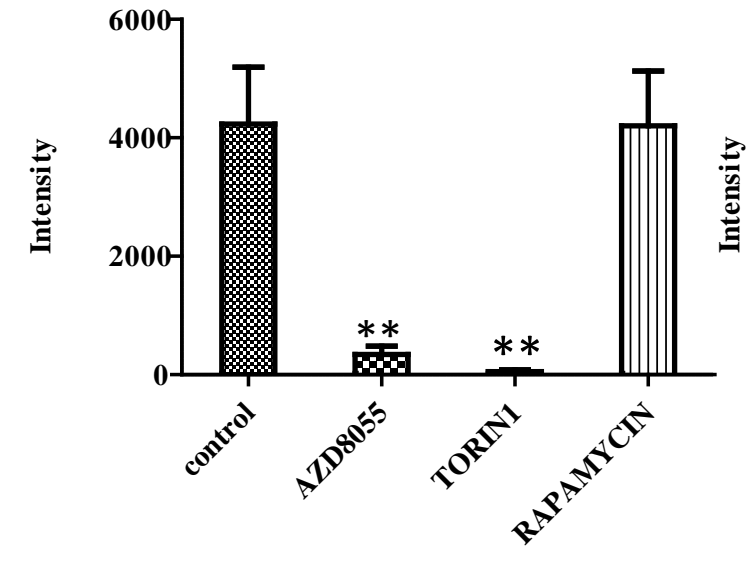
	Score	Expect	Method	Identities	Positives	Gaps
	316 bits(810)	1e-114	Compositional matrix adjust.	175/246(71%)	200/246(81%)	1/246(0%)
At RPS6	1		MKFNVANPTTGCQKKLEIDDDQKLRAFFDKRLSQEVSGDALGEEFKGYVFKIMGGCDKQG	60		
Cr RPS6	1		MK N+A P TGCQKKLE+DD+ KLRAF+D+R++ EV G+ LGEEFKGYV KI GG DKQG	60		
At RPS6	61		FPFKQGVLTFRVRLLLHRGTPCFRGGHRRRTGERRRKSVRGCIVSPDLSVLNLVIVKKGV	120		
Cr RPS6	61		F MKQGVLT RVRL+ G FRG+GRR GERRRKSVRGCIVSPDL+VLNLVIVKKG	120		
At RPS6	121		SDLPGLTDTEKPRMRGPKRASKIRKLFNLGKEDDVRKYVNTYRRTFTNKKGKKVSKAPKI	180		
Cr RPS6	121		+LPGLTD EKPR+RGPKRASKIRK+FNLGK DDVRKYV Y R T+K GKK K PKI	180		
At RPS6	181		QRLVTP LQQRARIADKKKRIAKANSDAADYQKLLASRLKEQRDRRSESLAKKRS-RL	239		
Cr RPS6	181		QRLVTP LQQRAR + KK++ K +DAA+Y KLL RLKEQR+RRSESLAKKR+ R+	240		
At RPS6	240	SSAPAK	245			
Cr RPS6	241	+S +K ASQASK	246			

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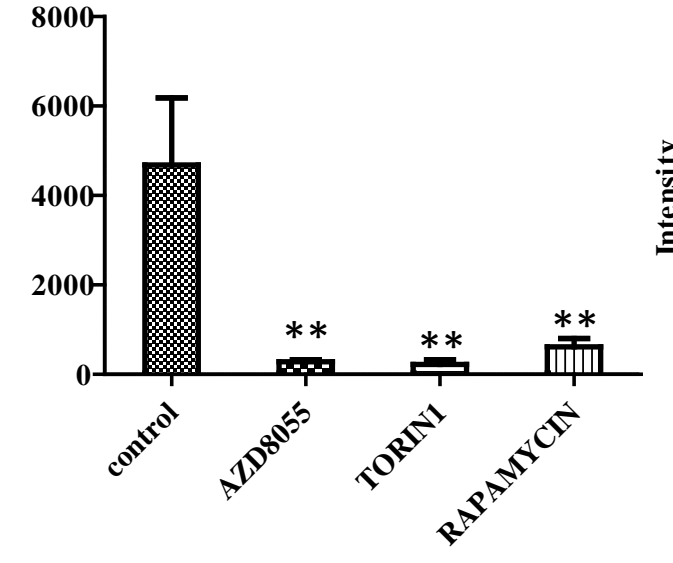
b.



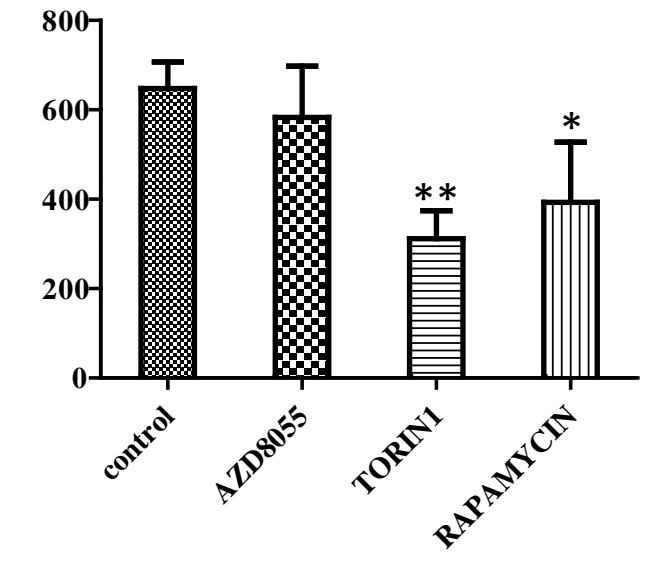
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LARP1



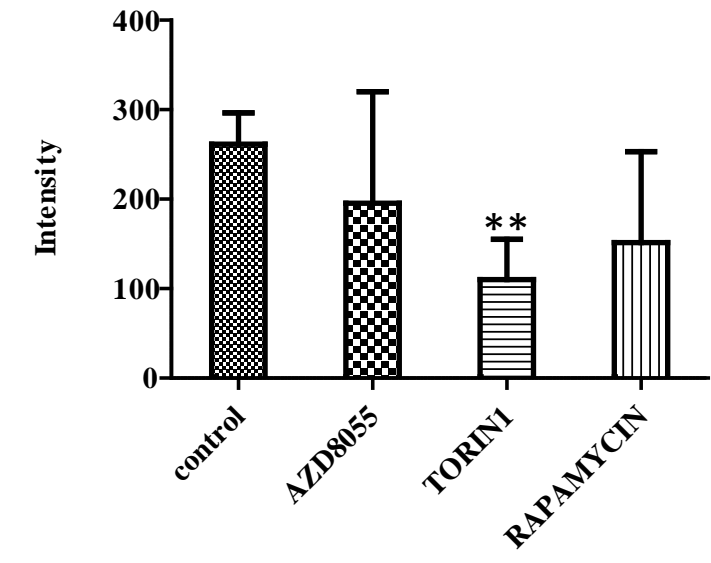
Cre10.G441200.T1.2--S817
LARP1



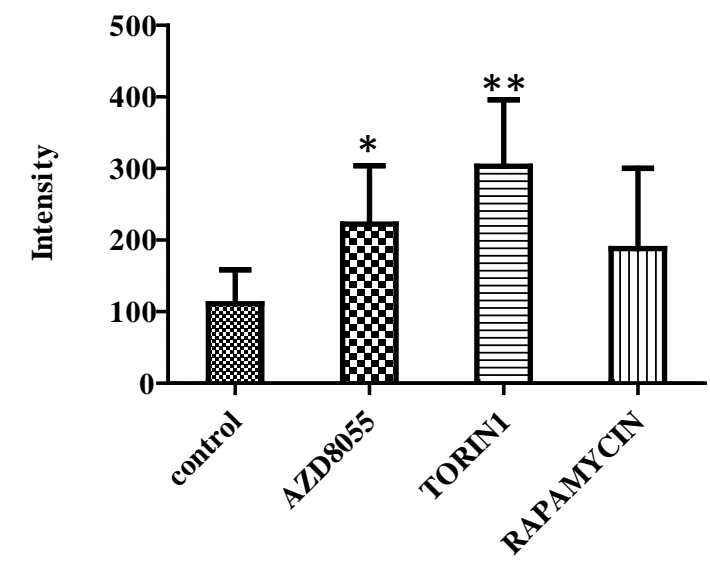
Cre09.g400650.t1.2--T127
RP-S6e, RPS6



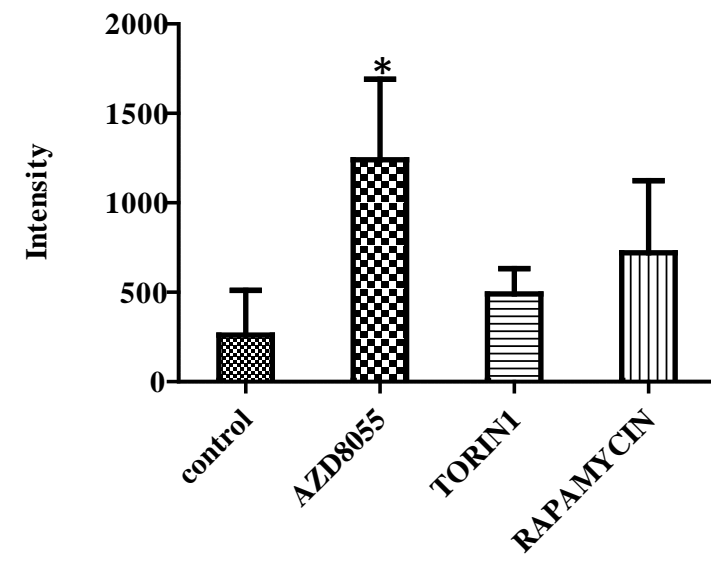
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EEF2K



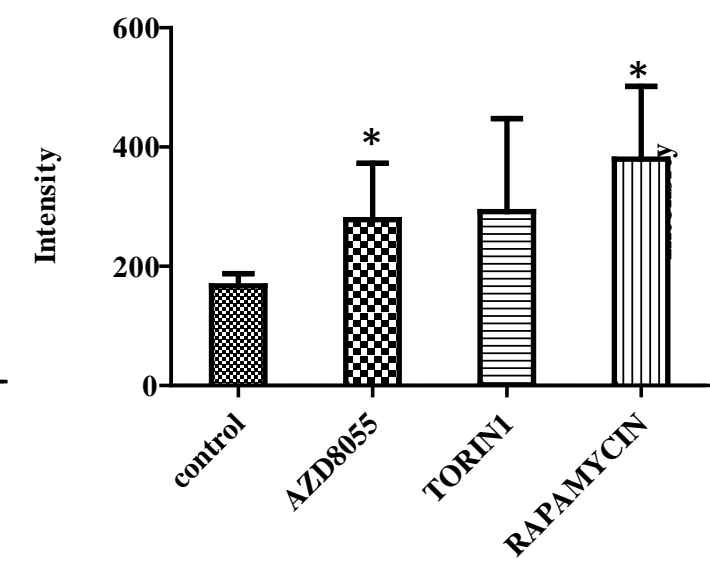
Cre17.g721850.t1.2--S853/S857:NL
EEF2K



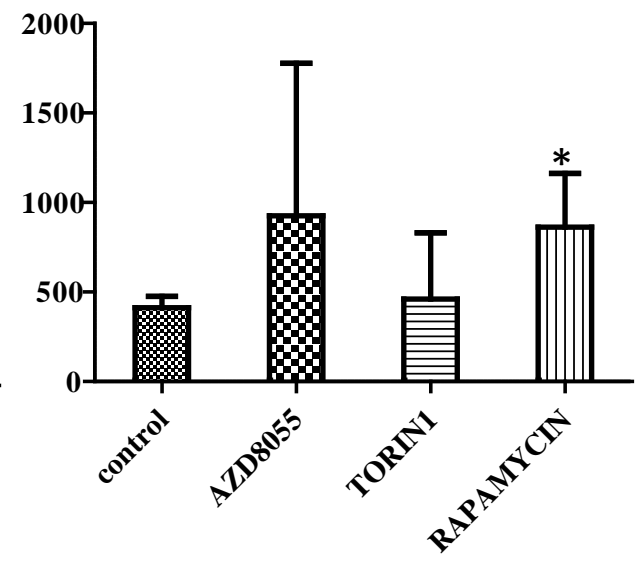
Cre12.g516200.t1.2--T57/T59:NL
EEF2



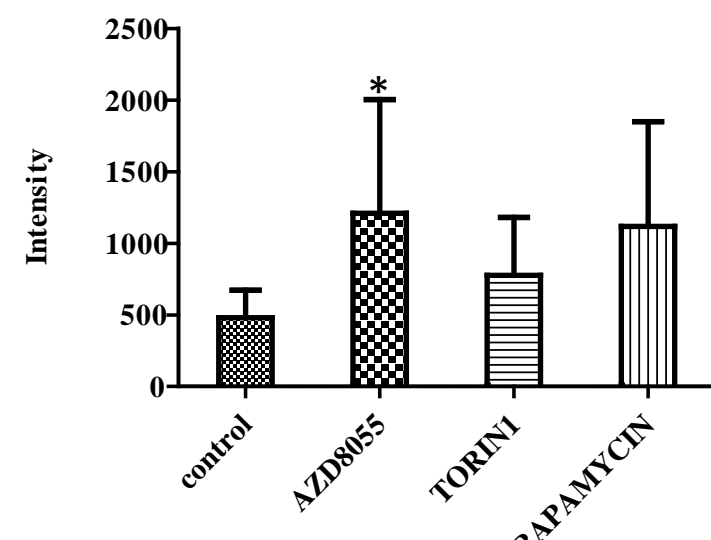
Cre10.g457500.t1.1--S25/S29:NL
PRKAB



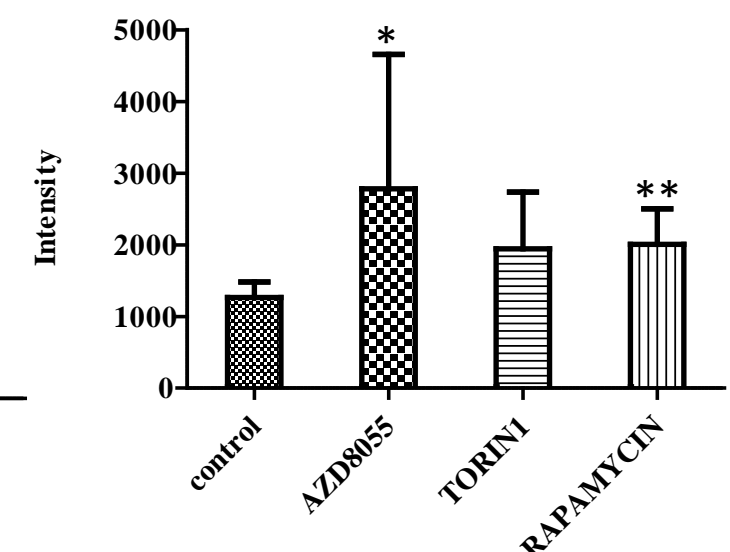
Cre02.g076350.t1.2--S8
ATP6B



Cre11.g468550.t1.2--S7/S8:NL
ATP6G



Cre02.g076900.t1.1--S71
PRKG1



Decrease in phosphorylation

Increase in phosphorylation

