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32 transferase (OGT); O-linked N-acetylglucosamine (O-GlcNAc); MYPT1 ; cell cycle ;
33 mitosis ; protein phosphorylation ; CDK1 ; PLK1 ; centrosome
34

35 **ABSTRACT**

36 The role of O-linked N-acetylglucosamine (O-GlcNAc) modification in the cell cycle
37 has been enigmatic. Previously, both O-GlcNAc transferase (OGT) and O-GlcNAcase
38 (OGA) disruption have been shown to derail the mitotic centrosome numbers,
39 suggesting that mitotic O-GlcNAc oscillation needs to be in concert with mitotic
40 progression to account for centrosome integrity. Here we attempted to address the
41 underlying molecular mechanism by both chemical approaches and biological assays,
42 and observed that Thiamet-G (OGA inhibitor) incubation strikingly elevated
43 centrosomal distances, suggestive of premature centrosome disjunction. These
44 aberrancies could be overcome by inhibiting Polo-like kinase 1 (Plk1), a mitotic
45 master kinase. Plk1 inactivation is modulated by the Myosin Phosphatase Targeting
46 Subunit 1 (MYPT1)-Protein Phosphatase 1 c β (PP1c β) complex. Interestingly,
47 MYPT1 is abundantly O-GlcNAcylated and the modified residues have been detected
48 in a recent O-GlcNAc profiling screen utilizing chemoenzymatic labeling and
49 bioorthogonal conjugation. We demonstrate that MYPT1 is O-GlcNAcylated at T577,
50 S585, S589 and S601, which antagonizes CDK1-dependent phosphorylation at S473,
51 subsequently attenuating the association between MYPT1 and Plk1, and promoting
52 PLK1 activity. Thus under high O-GlcNAc, Plk1 is untimely activated, conducive to
53 inopportune centrosome separation and disrupting the cell cycle. We propose that too
54 much O-GlcNAc is equally deleterious as too little O-GlcNAc, and a fine balance
55 between the OGT/OGA duo is indispensable for successful mitotic divisions.

56 INTRODUCTION

57 The centrosomes are the primary microtubule-organizing centers that nucleate
58 the mitotic spindle apparatus to ensure subsequent faithful sister chromatid
59 segregation during mitosis. The centrosome cycle is tightly coordinated with other
60 cell cycle events ¹, and its aberrancy could culminate in chromosome segregation
61 defects and aneuploidy ². The entire centrosome cycle encompasses centrosome
62 duplication during S phase, disjunction in late G2 and further separation during
63 prophase or prometaphase, and eventual segregation into the two daughter cells.

64 Centrosomes duplicate in concomitant with DNA replication, after which the
65 sister centrosomes are glued together by two proteinaceous linkers, c-Nap1 and
66 rootletin ^{3,4}, as well as other components such as Cep68, Cep215 and LRRC45 ⁵.
67 C-Nap1, a large coiled-coiled protein, links rootletin to the centrioles so that the
68 centrosome pair is joined by fibrous polymers ³. In late G2, the Never In Mitosis
69 (NIMA)-related serine/threonine kinase Nek2A phosphorylates and displaces c-Nap1
70 and rootletin, leading to disjoint centrosomes ⁶. Centrosomal accumulation of Nek2A
71 is mediated by the Hippo pathway, among which sterile 20-like kinase 2 (Mst2) and
72 Salvador (Sav1) play critical roles. In particular, Mst2 phosphorylates and activates
73 Nek2A ⁷. Upstream of Mst2 is the mitotic master kinase, Polo-like kinase 1 (PLK1) ⁸.

74 Following centrosome disjunction, the kinesin Eg5 accounts for centrosome
75 positioning in the beginning of M phase. Cyclin-dependent kinase 1 (Cdk1)
76 phosphorylates and activates Eg5 at T927 by stimulating the engagement between

77 Eg5 and microtubules⁹. Independently, centrosomal localization of Eg5 requires
78 PLK1¹⁰, which activates the NIMA-family kinase Nek9, leading to Eg5
79 phosphorylation at S1033 by the Nek9/6/7 complex¹¹. Phosphorylated Eg5 then binds
80 centrosomal Targeting Protein for Xenopus kinesin-like protein 2 (TPX2), which is
81 also mediated by Nek9¹². Besides mitosis, Eg5 also governs centrosome dynamics
82 during interphase¹⁰. Hence, the centrosomal role of Plk1 is two-fold : centrosome
83 disjunction via the PLK1-Mst2-Nek2A signaling cascade, and centrosome separation
84 through PLK1- Nek9/6/7- Eg5¹³.

85 Besides centrosomes, PLK1 also orchestrates a multitude of cell cycle events,
86 including replication, mitotic entry, chromosome segregation and cytokinesis^{4,14-16}. It
87 contains an N-terminal kinase domain and a C-terminal polo-box binding domain
88 (PBD). Phosphorylation of PLK1 at T210 at the T-loop is mediated by the Aurora
89 A-Bora complex¹⁷, resulting in dissociation of PBD from the kinase domain and thus
90 activating PLK1. Dephosphorylation of PLK1 is modulated by the protein
91 phosphatase 1 c β (PP1c β), targeted by the myosin phosphatase targeting subunit 1
92 (MYPT1)¹⁸. Specifically, CDK1 phosphorylates MYPT1 at Ser473, creating a
93 binding pocket between MYPT1 and the PBD of PLK1. Subsequently MYPT1
94 recruits PP1c β to dephosphorylate pT210 of PLK1¹⁸. Such interaction at the
95 kinetochore destabilizes kinetochore-microtubule attachments¹⁹. Besides
96 phosphorylation, PLK1 is also methylated at K209^{20,21}, which vies with pT210 and
97 hence blocking Plk1 activity.

98 Due to the vital role of PLK1 in mitosis, MYPT1 is subject to multifaceted
99 regulations: the Hippo pathway kinase LATS1/WARTS phosphorylates MYPT1 at
100 S445 to inactivate PLK1²²; optineurin, another phosphatase, promotes MYPT1
101 activity²³; checkpoint kinase 1 (CHK1) phosphorylates MYPT1 at S20, and
102 enhances MYPT1-PP1c β binding²⁴; checkpoint kinase 2 (CHK2) phosphorylates
103 MYPT1 at S507 to attenuate pS473²⁵.

104 Previous investigations have identified that MYPT1 is also subject to O-linked
105 N-acetylglucosamine (O-GlcNAc) modifications²⁶. O-GlcNAcylation is an emerging
106 post-translational modification (PTM) that integrates the metabolic signals with
107 transcription, nutrient sensing, stress responses and cell cycle events^{27,28}. It is
108 catalyzed by the sole transferase O-GlcNAc transferase (OGT), and reversed by the
109 only O-GlcNAcase (OGA)²⁷. Chemical inhibitors of OGT [acetyl-5S-GlcNAc (5S-G)]
110 and OGA [Thiamet-G (TMG)] have been developed to interrogate various biological
111 processes²⁹. During the cell cycle, O-GlcNAcylation levels fluctuate as the cells
112 proceed through different stages³⁰. In particular, both OGT and OGA overproduction
113 results in multipolar spindles³¹. However, myriad targets of O-GlcNAc and its
114 quintessential functions remain largely unexplored. Here we identify the O-GlcNAc
115 modified residues of MYPT1. We show that O-GlcNAcylation of MYPT1
116 antagonizes pS473, and results in its dissociation from PLK1. Elevated O-GlcNAc
117 levels thus fuel PLK1 activity towards centrosomes and render ill-timed centrosome
118 separation, disrupting the mitotic cell cycle.

119 **RESULTS**

120 **O-GlcNAc promotes aberrant centrosome separation via PLK1**

121 Previously, both OGT and OGA overproduction has been linked with multi-polar
122 spindle³¹. We sought to identify whether O-GlcNAc could also be linked with
123 centrosome dynamics. Strikingly, when HeLa cells were treated with TMG (OGAi),
124 the inter-centrosomal distance was significantly augmented four-fold (Fig. 1A),
125 reminiscent of the phenotype of Nek2A overexpression or over-activation^{4,32}. As the
126 centrosome cycle is tightly governed by PLK1, we attempted to inhibit PLK1. When
127 BI2536 (PLK1i) was adopted in conjuncture with TMG, the centrosomal distances
128 shortened considerably (Fig. 1A-C). When BI2536 was utilized alone (Fig. 1A), the
129 cells reduced centrosomal distances as previously reported⁴. These cytological
130 studies suggest that high O-GlcNAc culminates in premature centrosomal separation,
131 probably via PLK1.

132

133 **MYPT1 is O-GlcNAcylated at T577, S585, S589 and S601.**

134 Previous investigation has identified the inactivating phosphatase of PLK1 is
135 PP1c β , which is targeted by MYPT1¹⁸. Intriguingly, MYPT1 is O-GlcNAcylated²⁶.
136 Therefore we reasoned that O-GlcNAc might exert its effect through MYPT1.

137 First, we validated the interaction between MYPT1 and OGT through
138 biochemical assays. As shown in Fig. 2A, GST-OGT pulled-down HA-MYPT1 from
139 cell extracts. Then both OGT and MYPT1 proteins were purified from *E. coli*. Upon

140 incubation, His-OGT pulled-down GST-MYPT1 (Fig. 2B), suggesting that the
141 interact is direct.

142 Then we mapped which domain of MYPT1 interacts with OGT. As MYPT1 is a
143 relative large protein, we constructed several fragments of MYPT1 as previously
144 described: F1 (1-306), F2 (297-600), F3 (586-901) and F4 (886-1030) (Fig. 2C)²⁴. To
145 investigate which fragment interacts with OGT, recombinant full-length (FL) and
146 F1-F4 MYPT1 proteins were utilized in pulldown experiments, and the FL, F2 and F3
147 MYPT1 pulled-down Myc-OGT (Fig. 2D), suggesting that the potential modification
148 sites could be residing in F2 and F3.

149 A recent quantitative proteomic analysis of protein O-GlcNAc sites using an
150 isotope-tagged cleavable linker (isoTCL) strategy identified the potential O-GlcNAc
151 sites of MYPT1 to be T577, S585, S589 and S601³³ (Fig. 3 A-D), all of which locate
152 on F2 and F3. We constructed the T577A/S585A/S589A/S601A (4A) mutant
153 accordingly and assessed its effect. When HA-MYPT1-wild type (WT) and 4A
154 plasmids were transfected into cells, the 4A mutant significantly abrogated
155 O-GlcNAcylation (Fig. 4A), suggesting that these four amino acids are major
156 O-GlcNAc sites. Considering that MYPT1 is abundantly O-GlcNAcylated, and other
157 proteomic screens have also identified extra glycosylation sites³⁴, our results do not
158 exclude the possibility that there could be more O-GlcNAcylated residues on MYPT1.
159

160 **O-GlcNAcylation of MYPT1 antagonizes CDK1-dependent phosphorylation at**

161 **S473**

162 Since CDK1 phosphorylates MYPT1 at S473 during mitosis and creates a
163 binding motif between MYPT1 and the PBD of PLK1¹⁸, we surmised that O-GlcNAc
164 of MYPT1 might interplay with pS473. To address this possibility, we used a
165 phospho-specific antibody targeting pS473 that has been previously described and
166 utilized²⁵.

167 Then the WT and 4A plasmids are compared for the pS473 levels, and it is
168 significantly bolstered in the 4A mutant (Fig. 4B). When Noc was used to
169 synchronize cells in the M phase, O-GlcNAc levels decreased while pS473 levels
170 arose (Fig. 4C). As pS473 is mediated by CDK1, we adopted RO-3306 again, and
171 observed that RL2 levels decreased while pS473 levels increased in the
172 RO-3306-treated cells (Fig.4C). This is consistent with our conjecture that O-GlcNAc
173 antagonizes pS473. Lastly, we utilized the 5S-G inhibitor for OGT²⁹, and 5S-G
174 treatment substantially boosted pS473 levels of transfected HA-MYPT1(Fig. 4D). We
175 also examined the effects of 5S-G on endogenous MYPT1. Noc treatment enhanced
176 pS473 levels, and Noc plus 5S-G elevated pS473 markedly (Fig. 4E). In contrast,
177 glucose plus TMG (TMG+Glu) treatment during Noc would down-regulate pS473
178 compared to Noc alone (Fig. 4F). Taken together, O-GlcNAc of MYPT1 attenuates
179 pS473.

180

181 **O-GlcNAcylation inhibits MYPT1-PLK1 association**

182 Since pS473 promotes MYPT1-PLK1 association¹⁸, we then explored the effect
183 of O-GlcNAc on the interaction between MYPT1 and PLK1 by treating the cells with
184 TMG+Glu to enhance O-GlcNAc^{35,36}. As shown in Fig. 5A, Noc increased
185 PLK1-MYPT1 association discernably as reported¹⁸, but TMG+Glu together with
186 Noc obliterated PLK1-MYPT1 affinity.

187 As phosphorylated MYPT1 binds with PLK1-PBD¹⁸, we adopted GST
188 pulldown experiments using PLK1-PBD, and GST-PLK1-PBD modestly increased
189 binding with HA-MYPT1-4A (Fig. 5B). Then we employed FL-PLK1. When we
190 directly utilized the 4A mutant to coIP PLK1, the interaction between MYPT1 and
191 PLK1 substantially upregulated (Fig. 5C). When His-PLK1 was applied in pulldown
192 assays, 4A again manifested more robust association with PLK1 (Fig. 5D). In sum, the
193 binding between PLK1 and MYPT1 was abolished during high O-GlcNAc.

194

195 **O-GlcNAcylation of MYPT1 enhances PLK1 activity**

196 As the MYPT1 associates PLK1 to target PP1c β to dephosphorylate and
197 deactivate PLK1¹⁸, stronger affinity could signify less activity. We took advantage of
198 the IP-phosphatase assay to examine PLK1 activity^{22,24}. Cells were transfected with
199 Flag-MYPT1 and treated with Noc. Cells were also supplemented with TMG + Glu to
200 enrich for O-GlcNAc or not treated. When the anti-FLAG immunoprecipitates were
201 incubated with recombinant PLK1, the relative low O-GlcNAc group efficiently

202 dephosphorylated PLK1, as detected by IB with PLK1-pT210 antibodies, but not the
203 high O-GlcNAc group (Fig. 6A).

204 MYPT1-4A mutants were then directly exploited in the IP-phosphate assay. In
205 the absence of Noc, MYPT1-WT decreased PLK1 pT210 levels, and the MYPT1-4A
206 completely abolished PLK1-pT210 levels (Fig. 6B). This is consistent with our results
207 in Fig. 5C-D that MYPT1-4A partners with PLK1 in the absence of Noc treatment.
208 Collectively, our biochemical assays suggest that O-GlcNAcylated MYPT1 disjoins
209 PLK1 and promotes its kinase activity.

210

211 **MYPT1-4A Suppresses the TMG-induced centrosome disjunction defects**

212 Since the aforementioned results suggest that MYPT1 O-GlcNAcylation is a
213 pivotal regulator in centrosome separation, we undertook sh*MYPT1* to knockdown
214 endogenous MYPT1 (Fig. 7A), so that the effects of MYPT1-4A could be directly
215 measured and observed after TMG incubation. As shown in Fig. 7B, the premature
216 centrosome separation phenotype is discernable in the sh*MYPT1* cells that bears
217 MYPT1-WT plasmids. But in the cells transfected with MYPT1-4A plasmids, the
218 aberrancy is suppressed (Fig. 7C), in line with previous reports that PLK1
219 sequestration culminates in duplicated but unseparated centrosomes^{37,38}. Taken
220 together, the 4A mutant fails to show the untimely centrosome separation phenotype,
221 probably due to PLK1 suppression.

222

223

224 **DISCUSSION**

225 In this study, we identify that O-GlcNAc regulates centrosome separation
226 through the MYPT1-PLK1 complex. We pinpoint the major O-GlcNAcylated sites of
227 MYPT1 to be S564, S566, T570 and S578 in human cells, and further delineate that
228 O-GlcNAc antagonizes pS473, hinders MYPT1-PLK1 association, thus boosting
229 PLK1 activity and hence centrosome disjunction (Fig. 7D). When MYPT1 fails to be
230 O-GlcNAcylated, as in the MYPT1-4A mutant, PLK1 activity is dampened (Fig. 6B).

231 MYPT1 is one of the most abundant O-GlcNAcylated proteins, and its
232 modification sites have been unveiled time and again in distinct proteomic studies
233 ^{34,39,40}. Perhaps O-GlcNAc sites might not be conserved between humans and mice.
234 MYPT1 is found to be O-GlcNAcylated at S564, S566, T570 and S578 by mass
235 spectrometry in the mouse brain ⁴⁰, but our data show in HeLa cells O-GlcNAc occurs
236 in T577, S585, S589 and S601. Previously, p53 is identified to be O-GlcNAcylated at
237 S149 ⁴¹, which is not conserved in mice either. The same also holds true for
238 phosphorylation. For instance, Ataxia-telangiectasia mutated (ATM), a vital sensor
239 protein for DNA damage signaling, is phosphorylated at S1981 and then activated in
240 humans, but mutation of S1987 (the mouse equivalent) does not hinder ATM function
241 in mice ^{42,43}. Thus extrapolating data across species needs extra caution, as the
242 function and sites of PTMs could be context-dependent.

243 Along the same vein, there could be more O-GlcNAc sites on MYPT1, as the
244 proteomic studies were carried out under disparate circumstances and using different

245 click chemistry methodologies^{33,34,39,40}. As the O-GlcNAc modification is highly
246 dynamic, distinct sites could be modified in response to environmental cues.
247 O-GlcNAc could have multi-faceted effects on the centrosome. Both OGT and
248 OGA overexpression result in multi-polar spindles, which could be repressed by TMG
249 treatment³¹. NuMA (nuclear mitotic apparatus protein) is indispensable for spindle
250 pole formation and regulates spindle pole cohesion^{44,45}. NuMA is O-GlcNAcylated
251 and its localization was led astray by OGT overexpression⁴⁶. Further investigations
252 reveal that O-GlcNAcylated NuMA interacts with Galectin-3, which is a prerequisite
253 for mitotic spindle cohesion and proper NuMA localization⁴⁷. Here we reveal that
254 centrosome dynamics is also governed by O-GlcNAcylation levels. As the
255 centrosome is pivotal for the mitotic process, O-GlcNAc is bound to modulate other
256 aspects of centrosome function.

257 Our results indicate that O-GlcNAcylated MYPT1 attenuates interaction with
258 PLK1 and thus promotes PLK1 activity. It is intriguing that overall PLK1 pT210
259 levels remain unaltered in OGT or OGA overproduction cells^{31,46}. We did not detect
260 discernable difference either, in cells supplemented with TMG plus Glucose (data not
261 shown). This may seem paradoxical at first, but considering the versatile roles of
262 PLK1 during mitosis^{4,14-16}, we could entertain the possibility that only a small pool of
263 PLK1 is regulated by MYPT1. First, although the overall activity of PLK1 is
264 upregulated during mitosis, CDK1 actually dampens PLK1 activity via MYPT1 in a
265 mitosis-specific fashion¹⁸. Secondly, the pool of PLK1 responsible for

266 kinetochore-microtubule attachment actually contains low PLK1 kinase activity
267 during metaphase so that microtubules could be dynamic⁴⁸. Therefore, irrespective of
268 the overall elevation of mitotic activity, the mitotic master kinase - PLK1 is perhaps
269 indeed fine-tuned in space and time. And O-GlcNAc, could be the sweet icing on the
270 cake.

271

272 **EXPERIMENTAL PROCEDURES**

273 *Cell culture, antibodies and plasmids*

274 Cells were purchased from ATCC. Antibodies were as follows: anti-MYPT1
275 (Bethyl, # BL3866), anti-PLK1 (Zymed, #37-7100), PLK1-pT210 (BD Pharmingen,
276 #558400). Antibodies against pS473 were prepared as described before²⁵ and
277 manufactured by Beijing B&M Biotech Co., Ltd.. *MYPT1* plasmids were described
278 before⁴⁹. *MYPT1-4A* plasmids were generated using specific primers (sequences
279 available upon request) following the manufacturer's instructions (QuickChange II,
280 Stratagene). His-OGT was from Dr. Yue Wang (Peking Univ.). The following
281 shRNA target sequences were used: sh*MYPT1*: GTAACCCAGTGGACCATAATT.

282

283 *Immunoprecipitation (IP) and Immunoblotting (IB) assays*

284 IP and IB experiments were performed as described before⁵⁰. The following
285 primary antibodies were used for IB: anti- β -actin (1:10000), anti-HA (1:1000), and
286 anti-FLAG M2 (Sigma) (1:1000), anti-Myc (1:1000), anti-PLK1 (1:1000),

287 anti-MYPT1 (1:1000), PLK1-pT210 (1:500). The IP-phosphatase assay was
288 performed as before^{22,24}.

289 Peroxidase-conjugated secondary antibodies were from JacksonImmuno
290 Research. Blotted proteins were visualized using the ECL detection system
291 (Amersham). Signals were detected by a LAS-4000, and quantitatively analyzed by
292 densitometry using the Multi Gauge software (Fujifilm). All western blots were
293 repeated for at least three times.

294

295 *Cell Culture Treatment*

296 Chemical utilization: Nocodazole (Noc) at 100 ng/ml for 16 hours; Ro 3306
297 (CDK1 inhibitor) at 2 μ M for the time indicated; BI2536 (PLK1 inhibitor) at 100 nM
298 for two hours; Thiamet-G (TMG) (OGA inhibitor) at 5 μ M for 24 hrs ;
299 acetyl-5S-GlcNAc (5S-G) (OGT inhibitor) was used at 100 μ M (prepared at 50 mM in
300 DMSO) for 24 hrs [64].

301

302 *Indirect Immunofluorescence*

303 Indirect immunofluorescence staining was performed as described before⁵⁰.

304 Dilutions of primary antibodies were 1:1,000 for mouse anti- γ -tubulin. Cell nuclei
305 were stained with DAPI. Quantitation was performed with the software Image J.

306

307 **Abbreviations:**

308 PTM, post-translational modification; O-GlcNAc, O-linked N-acetylglucosamine;
309 OGT, O-GlcNAc transferase; TMG, Thiamet-G; PBD, polo-box binding domain ;
310 MYPT1, myosin phosphatase targeting subunit 1; Cdk1, Cyclin-dependent kinase 1 ;
311 PLK1, Polo-like kinase 1 ; 5S-G, acetyl-5S-GlcNAc; PP1c β , protein phosphatase 1 c
312 β ; FL, full-length ; ETD, Electron Transfer Dissociation

313

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324

325 **Competing interests**

326 The authors declare that they have no conflicts of interest with the contents of this
327 article.

328

329 **Author contributions**

330 J. L., X.C. and Z.T. conceived the project and analyzed the data. C. L., Y. S., X. L., Z.

331 X. and Jie L. performed all the experiments. All authors reviewed and approved the

332 manuscript.

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490

491

492 **Figure Legends**

493 **Figure 1. Elevated O-GlcNAc levels leads to aberrant centrosome separation via**

494 **PLK1.** (A) HeLa cells were treated with TMG, BI2536 or TMG + BI2536, then

495 stained with anti- γ -tubulin antibodies and DAPI. Scale bar, 10 μ M. (B) Quantitation

496 of inter-centrosomal distances in (A). More than 25 cells were counted for each

497 experiment. The data represent mean \pm S. D. of three independent experiments.

498 Asterisks indicate significant difference as determined by t-test (p1-2=0.005,

499 p2-4=0.008). (C) Quantitation of percent of cells with separated centrosomes in (A).

500 Asterisks indicate significant difference as determined by t-test (p1-2=0.02,

501 p2-4=0.02)

502

503 **Figure 2. OGT interacts with the central region of MYPT1.** (A) Recombinant

504 GST-OGT proteins were incubated with HA-MYPT1-transfected cell lysates. (B)

505 His-OGT and GST-MYPT1 proteins were incubated together and then subject to

506 pulldown assays as indicated. (C) A diagram showing MYPT1 constructs used in this

507 study. Full-length (FL), F1(1-306), F2(297-600), F3(586-901) and F4(886-1030) were

508 previously described²⁴. 4A denotes T577AS585AS589AS601A. (D) Recombinant

509 GST-MYPT1-FL, F1, F2, F3 and F4 proteins were purified from bacteria, and

510 incubated with extracts from 293T cells transfected with Myc-OGT. Asterisks

511 demarcate corresponding proteins.

512

513 **Figure 3. MYPT1 is O-GlcNAcylated at T577, S585, S589 and S601. (A-D)**

514 Electron Transfer Dissociation (ETD) mass spectrometry combined with
515 chemo-enzymatic labeling identified that T577S585 S589S601 are O-GlcNAcylated
516 ³³.

517

518 **Figure 4. O-GlcNAcylation of MYPT1 antagonizes CDK1-dependent**

519 **phosphorylation at S473.** (A) MYPT1-WT and 4A plasmids together with
520 Myc-OGT or empty vectors were transfected into 293T cells and then blotted with
521 antibodies indicated. (B) Cells were transfected with HA-MYPT1-WT or 4A plasmids,
522 and then the lysates were IBed with antibodies indicated. (C) Cells were treated with
523 Noc, or Noc with Ro-3306 for the time indicated. (D) HeLa cells were transfected
524 with HA-MYPT1, treated or untreated with 5S-G (OGT inhibitor). (E) Cells were
525 treated with Noc, or Noc + 5S-G. (F) Cells were transfected with MYPT1-WT
526 plasmids, and then treated with Noc, or Noc plus TMG + Glu as indicated.

527

528 **Figure 5. O-GlcNAcylation of MYPT1 attenuates the interaction between**

529 **MYPT1 and PLK1.** (A) 293T cells were transfected with Flag-PLK1 and
530 HA-MYPT1, treated or not treated with Noc, TMG + Glu, respectively, then subject
531 to IP and IB as indicated. (B) GST-PLK1-PBD proteins were purified from bacteria.
532 Cells were transfected with HA-MYPT1-WT or 4A, then the cell lysates were subject
533 to GST-PLK1-PBD pulldown assays. (C) Cells were transfected with FLAG-PLK1

534 together with HA-MYPT1-WT or 4A, then subject to IP and IB as indicated. (D)

535 Cells were transfected with HA-MYPT1-WT or 4A, and then cell extracts were

536 utilized in His-PLK1 pulldown assays.

537

538 **Figure 6. O-GlcNAcylation of MYPT1 promotes PLK1 activity.** (A)

539 IP-phosphatase assays. U2OS cells were transfected with Flag-MYPT1, synchronized

540 to mitosis with Noc, then treated with TMG + Glu or left untreated. The anti-Flag

541 immunoprecipitates were then incubated with recombinant His-PLK1. (B)

542 IP-phosphatase assays using the MYPT1-WT and -4A mutants without Noc

543 treatment.

544

545 **Figure 7. MYPT1 overproduction overrides the centrosome disjunction defects**

546 **induced by TMG** (A) Lenti viruses encoding vectors or sh*MYPT1* was introduced

547 into HeLa cells, together with HA-MYPT1-WT or -4A plasmids. The cellular lysates

548 were IBed with the antibodies indicated. (B) Cells in (A) were subject to indirect IF

549 using the antibodies indicated. (C) Quantitation of percent of cells with separated

550 centrosomes in (B). Asterisks indicate significant difference as determined by t-test

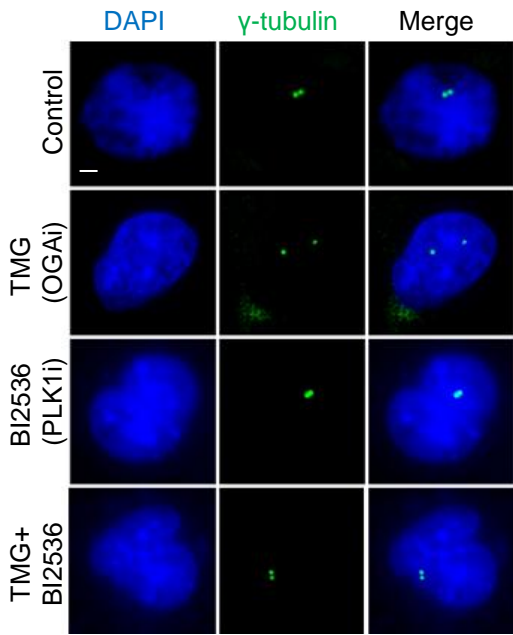
551 ($p_{1-2}=0.0002$, $p_{2-3}=0.22$, $p_{2-4}=0.001$). (D) We propose that MYPT1 is

552 O-GlcNAcyated at T577 S585 S589 S601, which antagonizes CDK1-dependent

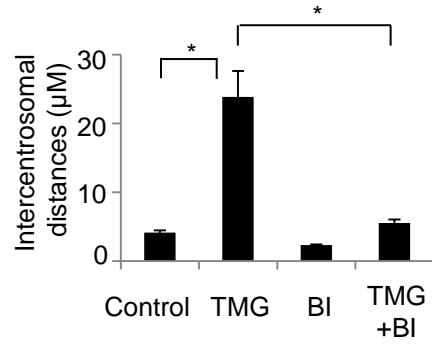
553 phosphorylation at S473 and MYPT1-PLK1 interaction. By disjoining PLK1 from the

554 MYPT1/PP1c β complex, PLK1 activity is elevated, thus promoting centrosome
555 separation.

A



B



C

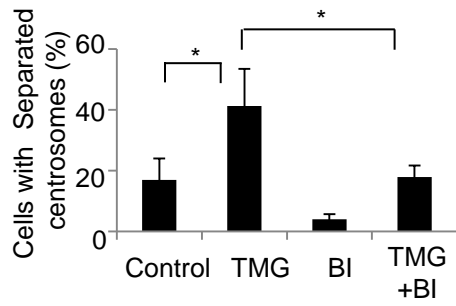


Figure 1

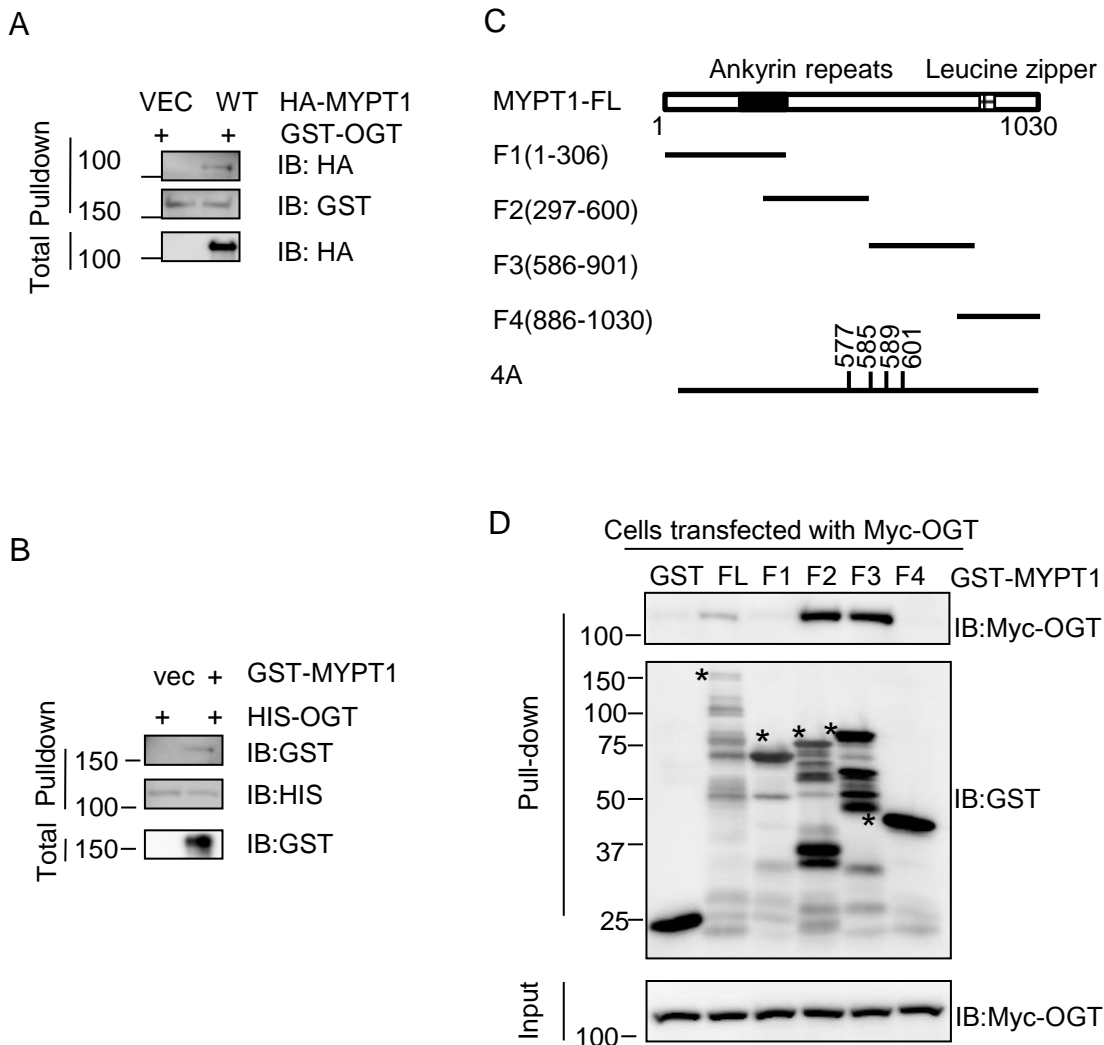
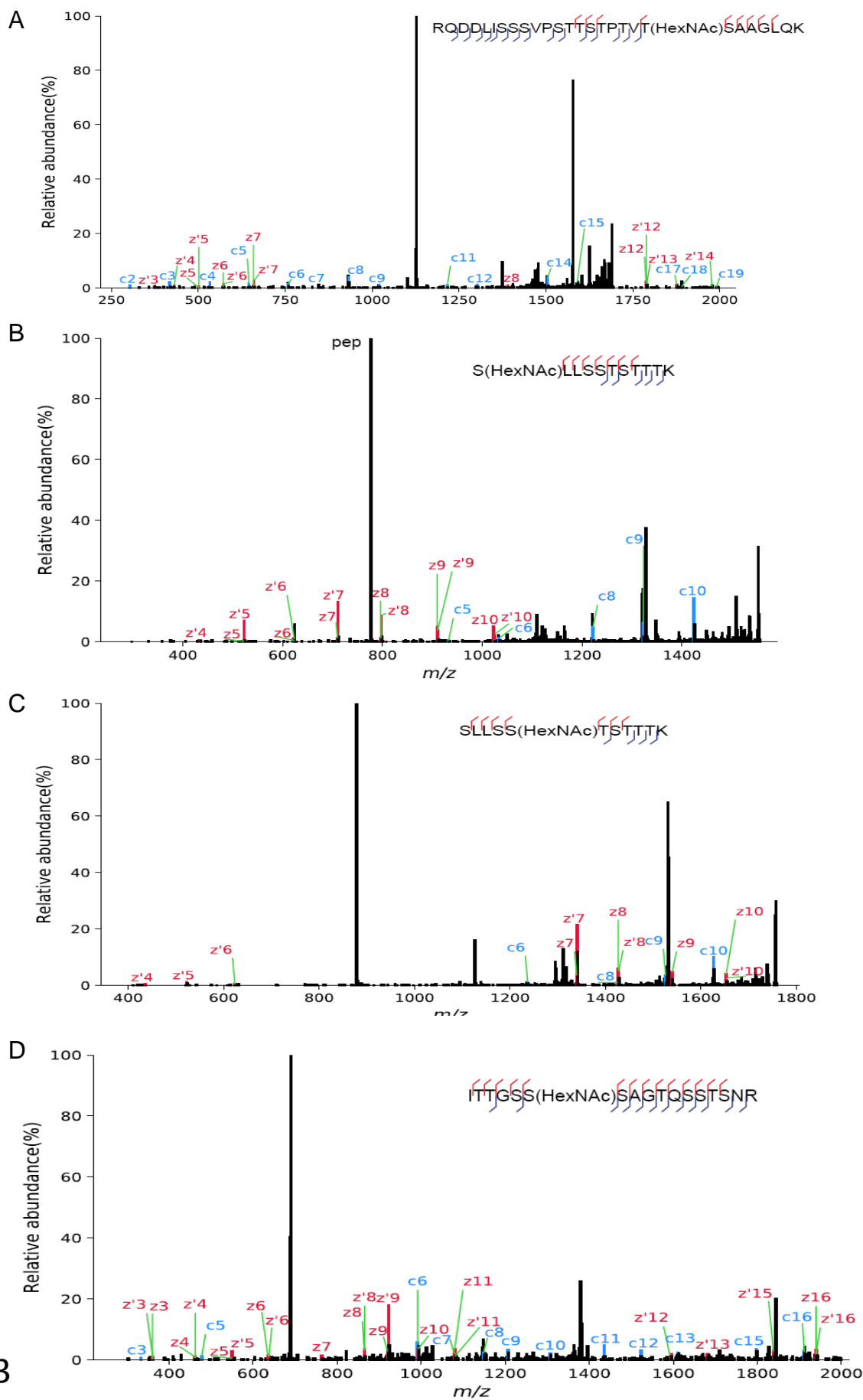


Figure 2



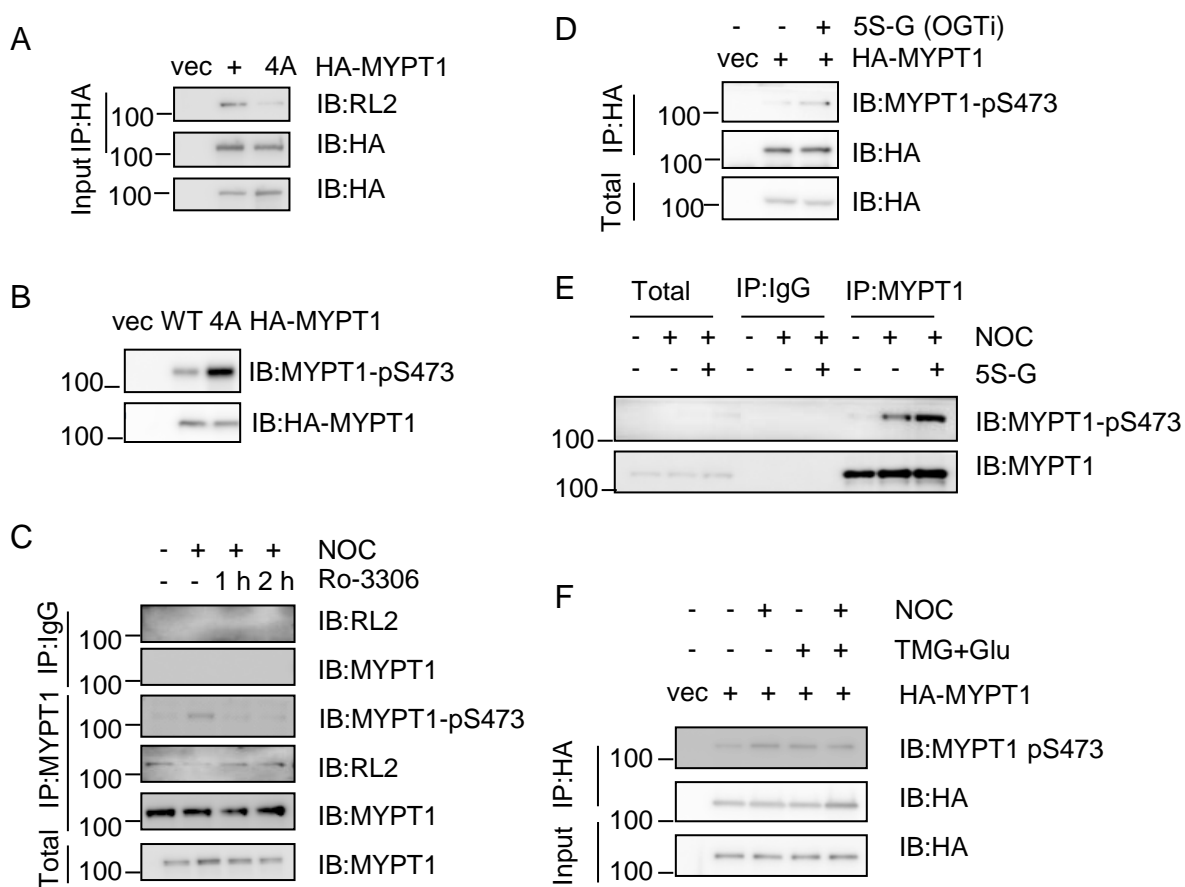


Figure 4

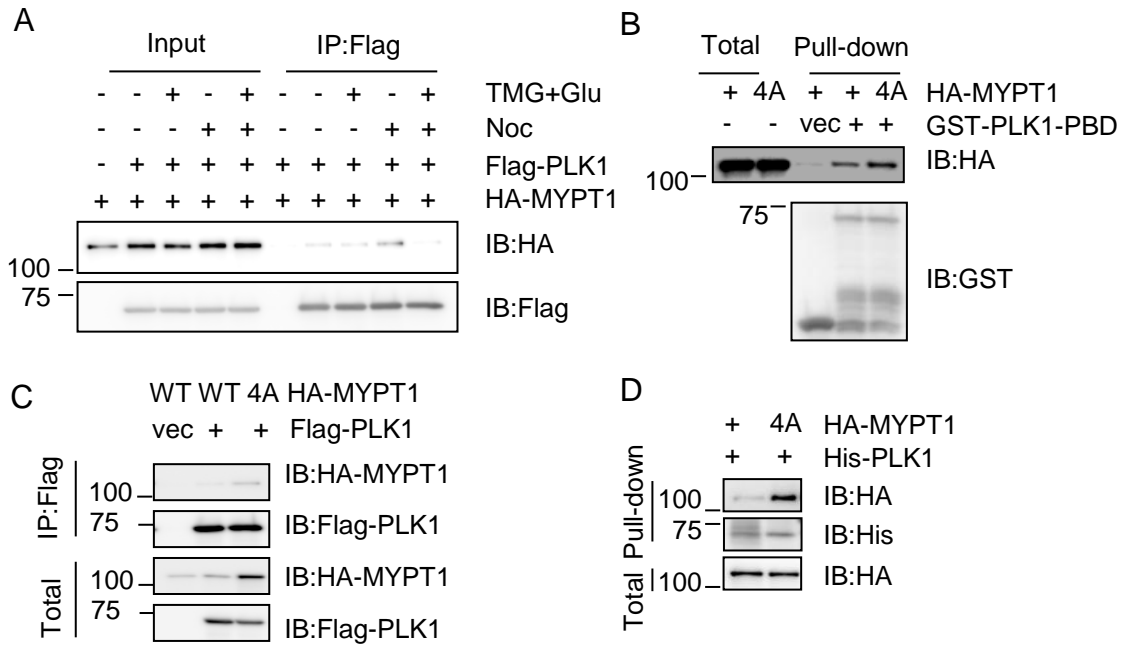


Figure 5

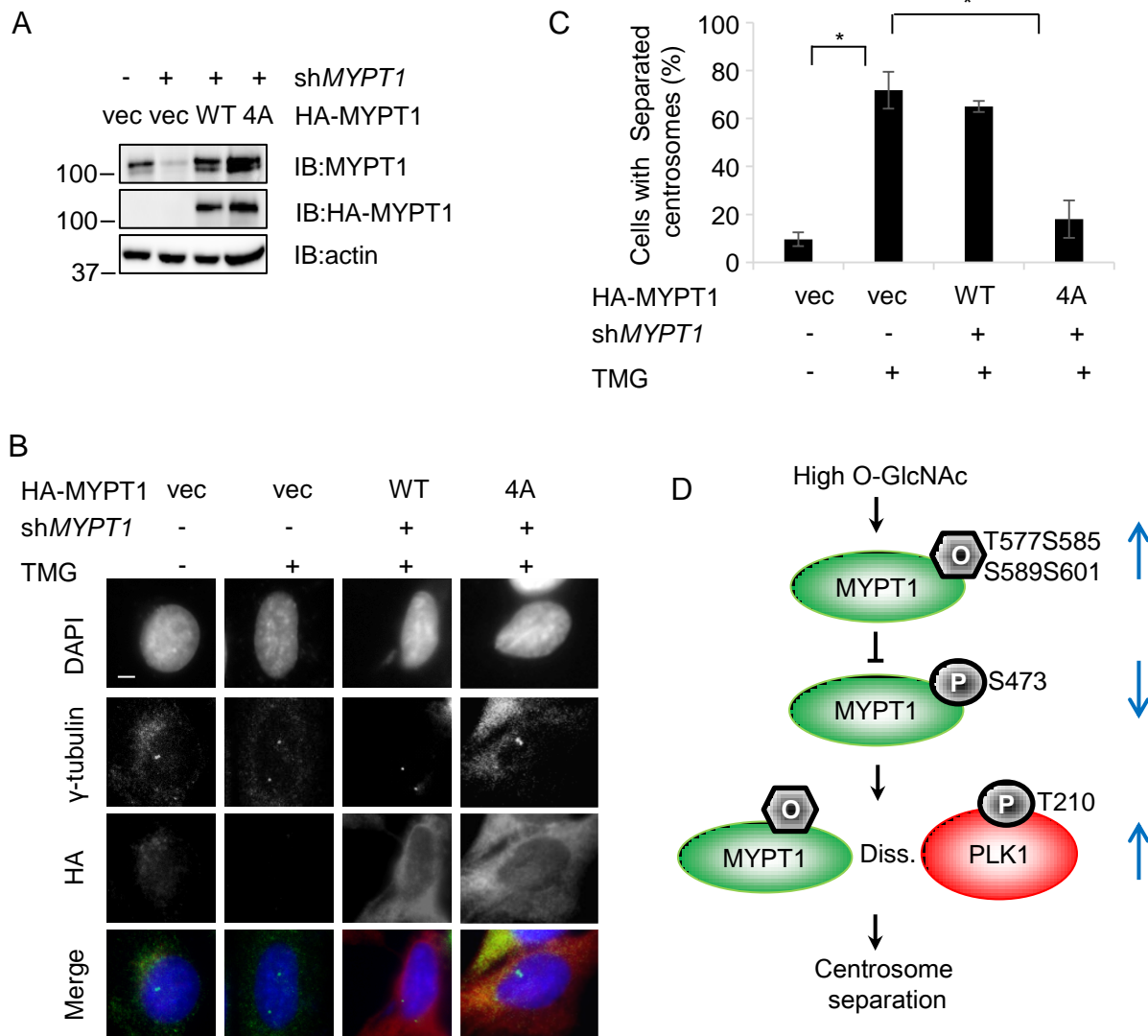


Figure 7