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Thymidylate Synthase O-GlcNAcylation: a molecular mechanism of 5-FU sensitization in colorectal cancer

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25

26 **Abstract**

27 Alteration of *O*-GlcNAcylation, a dynamic post-translational modification, is associated with tumorigenesis and
28 tumor progression. Its role in chemotherapy response is poorly investigated. Standard treatment for colorectal
29 cancer (CRC), 5-fluorouracil (5-FU), mainly targets Thymidylate Synthase (TS). TS *O*-GlcNAcylation was reported
30 but not investigated yet. We hypothesize that *O*-GlcNAcylation interferes with 5-FU CRC sensitivity by
31 regulating TS. *In vivo*, we observed that combined 5-FU with Thiamet-G (*O*-GlcNAcase (OGA) inhibitor)
32 treatment had a synergistic inhibitory effect on grade and tumor progression. 5-FU decreased *O*-GlcNAcylation
33 and, reciprocally, elevation of *O*-GlcNAcylation was associated with TS increase. *In vitro* in non-cancerous and
34 cancerous colon cells, we showed that 5-FU impacts *O*-GlcNAcylation by decreasing *O*-GlcNAc Transferase
35 (OGT) expression both at mRNA and protein levels. Reciprocally, *OGT* knockdown decreased 5-FU-induced
36 cancer cell apoptosis by reducing TS protein level and activity. Mass spectrometry, mutagenesis and structural
37 studies mapped *O*-GlcNAcylated sites on T251 and T306 residues and deciphered their role in TS proteasomal
38 degradation. We reveal a crosstalk between *O*-GlcNAcylation and 5-FU metabolism *in vitro* and *in vivo* that
39 converges to 5-FU CRC sensitization by stabilizing TS. Overall, our data propose that combining 5-FU-based
40 chemotherapy with Thiamet-G could be a new way to enhance CRC response to 5-FU.

41

42 **Introduction**

43 Thymidylate Synthase (TS) is a key ubiquitous enzyme involved in the *de novo* biosynthesis of
44 2'-deoxythymidine-5'-monophosphate (dTMP) and dihydrofolate (DHF) from
45 2'-dexoyuridine-5'-monophosphate (dUMP) and 5,10-methylenetetrahydrofolate (5,10-MTHF). dTMP is an
46 essential precursor for DNA synthesis and repair. TS expression and activity are increased in cancer to support
47 high cell proliferation rate, hence its inhibition is used in therapeutic strategies of several cancers including
48 colorectal cancer (CRC) (1,2). 5-fluorouracil (5-FU) is the gold standard treatment for CRC. In cells, 5-FU is
49 converted into the active metabolite 5-fluoro-2'-deoxyuridine monophosphate (FdUMP) which forms an

50 inactive complex with TS and 5,10-MTHF. This irreversible inhibition induces an accumulation of dUTP and a
51 depletion of 2'-deoxythymidine-5'-triphosphate (dTTP) leading to DNA damage and cell cycle arrest. Resistance
52 to 5-FU chemotherapy remains a challenge in the management of CRC and clinical outcome remains poor in
53 patients with advanced cancers. High TS expression levels and single nucleotide polymorphism in TS gene
54 (*TYMS*) are clinical predictive biomarkers of 5-FU-based chemotherapy resistance in CRC. However, numerous
55 clinical studies on *TYMS* expression and polymorphism in human cancers to clarify its significance as a
56 determinant of tumors 5-FU sensitivity do not appear consistent due to the variability in the genetic
57 backgrounds of the patients (3,4). Adequate amount of TS appears to be necessary for 5-FU sensitivity.
58 Post-translational modifications (PTMs) regulate enzymatic activity, nuclear translocation and degradation of
59 TS. TS is phosphorylated (5,6), SUMOylated (7), *N*^α-acetylated (8), ubiquitinated (8) and *O*-GlcNAcylated (9,10).
60 However, the role of these PTMs in the 5-FU response remains unknown. *O*-GlcNAcylation is a dynamic PTM
61 finely tuned by two antagonistic enzymes: *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA) that
62 respectively transfers and hydrolyzes the *O*-GlcNAc moiety on serine and threonine residues of proteins in
63 response to cell nutrient state. This PTM regulates interactions, stability, enzymatic activity and subcellular
64 localization of target proteins, hence a plethora of fundamental cellular mechanisms (11). Alteration of
65 *O*-GlcNAcylation was reported in many cancers and several studies pointed out its involvement in the etiology
66 and progression of the disease (12). Emerging research demonstrates that *O*-GlcNAcylation could also regulate
67 the response of cancerous cells to therapeutic drugs such as tamoxifen (13), Tumor necrosis factor related
68 apoptosis-inducing ligand (TRAIL) therapy (14,15), cisplatin (16–18), bortezomib (19,20), doxorubicin (21) and
69 5-FU (22). Herein, we report multiple lines of *in vivo* and *in vitro* data supporting the *O*-GlcNAcylation
70 sensitizing effect to 5-FU response in CRC, involving the novel finding that this PTM at Thr²⁵¹ and Thr³⁰⁶
71 stabilizes TS.

72

73 **Materials and Methods**

74 **Cell lines**

75 CCD 841 CoN (CRL-1790) and parental HT-29 (HTB-38), purchased from American Type Culture Collection
76 (ATCC), and 5-FU resistant HT-29 5F31 cells (23) were cultured respectively in EMEM with 5 mM glucose,
77 McCoy's 5A and DMEM with 25 mM glucose (Lonza) supplemented with 10% heat inactivated FBS (Dutscher)
78 and 2 mM L-glutamine. Cells were seeded or transfected in their respective medium and then, 24 h later,
79 grown in DMEM with 25 mM glucose (High Glucose, HG) unless stated otherwise in figure legend. For
80 transfection procedures see supplemental material.

81

82 **Animal models**

83 Animal care and procedures were carried out according to the French guidelines (APAFIS#1879-
84 2018121918307521) by the Ethics Committee on Animal Experiments (CEEA) 75 Hauts-de-France. Forty-five
85 C57BL/6J OlaHsd wild-type age-matched (8 weeks) male mice (Envigo Harlan) were housed in groups of 10 per
86 cage using a 12 h light/12 h dark cycle and provided with water and standard diet (SAFE) *ab libitum*. Forty mice
87 were injected intraperitoneally with 10 mg/kg AOM (Sigma-Aldrich) and 5 mice received an injection of a
88 vehicle (NaCl 0.9%) (untreated, healthy controls). The AOM-injected mice were given 3 cycles of DSS (MP
89 Biomedicals™) as follow: DSS 2.5% for 7 days in week 1, DSS 1.5% for 5 days in week 3 and DSS 1.5% for 5 days
90 in week 8. CRC induction was monitored under anesthesia and colonoscopies were performed at day 68 using a
91 high-resolution Karl Storz colonoscope (Tuttlingen). At day 73, AOM-injected and control mice were
92 randomized in 4 groups: vehicle (NaCl 0.9%), 5-FU (12.5 mg/kg/day) (Sigma-Aldrich), Thiamet-G (90 mg/kg/day)
93 and 5-FU + Thiamet-G. ALZET® osmotic pumps were subcutaneously implanted under anesthesia with a
94 connected ALZET® intraperitoneal catheter (Charles River) for continuous drug delivery at 0.5 µL/h for 13 days.
95 At day 86, tumor numbers and grade were assessed by colonoscopy (24). After sacrifice, colons were dissected,
96 flash-frozen and stored at -80°C or fixed in 10% buffered formalin for paraffin embedment.

97

98 **Plasmids**

99 See **Supplemental Experimental Procedures and Supplemental Table S1.**

100

101 **Cell growth assay and IC₅₀ determination**

102 Cells were seeded in a 96-well microplate 24 h prior treatment with increasing concentrations of 5-FU (0 to 20
103 μM for CCD 841 CoN and HT-29 cells, 0 to 50 mM for HT-29 5F31) for 48 h. Cell viability was analyzed using
104 CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) following the manufacturer's
105 instructions. Analysis of siOGT effect on the 5-FU IC₅₀ value was monitored on 24 h siOGT or siControl
106 transfected cells that were then incubated 72 h with a range of 5-FU (0 to 80 μM for CCD 841 CoN and HT-29
107 cells, 0 to 50 mM for HT-29 5F31). The cell viability was assessed in the same way.

108

109 **Cell cycle analysis**

110 Cells were harvested using trypsin/EDTA, washed twice with Phosphate-buffered saline (PBS) and fixed. Cells
111 were then washed twice with PBS and incubated for 1 h at 37°C with 10 ng/mL RNase A (Sigma-Aldrich) and
112 500 μg/mL propidium iodide (Sigma-Aldrich). DNA content was analyzed using FACScalibur cytometer (BD
113 Biosciences).

114

115 **SDS-PAGE and Western Blot**

116 See **Supplemental Experimental Procedures**.

117

118 **Immunoprecipitation and co-immunoprecipitation**

119 See **Supplemental Experimental Procedures**.

120

121 **Click chemistry**

122 Two-hundred micrograms of total proteins was methanol/chloroform precipitated and subjected to
123 Click-It™ O-GlcNAc Enzymatic Labelling System (Thermo Fisher Scientific) following the manufacturer's

124 instructions. GalNAz labeled samples (50 µg) were chemically labeled with 10 mM homemade DBCO-PEG mass
125 tag (4.4 kDa) as previously described (25).

126

127 **Real time RT-qPCR**

128 See **Supplemental Experimental Procedures and Supplemental Table S1.**

129

130 **Mass spectrometry**

131 Following SDS-PAGE of immunopurified TS and Coomassie Brilliant Blue staining, the TS was in-gel digested
132 with trypsin, and released peptides were analyzed by high resolution mass spectrometry according to a
133 previously published protocol (25) and further details in **Supplemental Experimental Procedures.**

134

135 **TS activity**

136 TS activity assay was measured according to the tritium-release assay (26) after cytosol incubation with 0.375
137 µCi [5-³H]-dUMP (Isobio) and 0.62 mM CH₂FH₄ (Santa Cruz). Radiolabeled ³H₂O was measured by Ultima Gold™
138 liquid scintillation (PerkinElmer) with a Hidex 300 SL scintillation counter. Cellular and intrinsic activity of TS
139 were respectively normalized using total proteins and TS proteins levels.

140

141 **Immunohistochemistry**

142 Immunohistochemistry staining and quantification on formalin fixed colon tissues were realized as we
143 previously described (27). Antigen retrieval on deparaffinized and rehydrated 4 µm histological sections was
144 performed in Tris-HCl 10 mM, EDTA 1 mM, pH 9 for anti-TS antibody [EPR4545] or Tris sodium citrate 10 mM
145 pH 6 for anti-O-GlcNAc antibody. The rabbit anti-TS [EPR4545] (1:100) or the anti-O-GlcNAc (1:1,000) antibody
146 were incubated overnight at 4°C.

147

148 ***In silico* modeling of TS structure**

149 Tridimensional structures of the human TS were retrieved from the Protein Data Bank (www.rcsb.org) under
150 the PDB codes 1HZW (X-ray diffraction at 2.0 angstroms resolution) and 1I00 (X-ray diffraction at 2.5 angstroms
151 resolution) (28). Missing sequences M1-G29 and I307-V313 were built and added to the X-ray structure 1HZW.
152 A conformational analysis of the whole structure was performed keeping the E30-T306 as aggregate. Once the
153 amino acids under study were modified, they were optimized within the protein using a classical Monte Carlo
154 conformational searching procedure as described in the BOSS software (29). An empirical potential energy was
155 further evaluated for the various post-translationally modified TS using the SPASIBA force field and the
156 corresponding parameters, in particular for proteins and saccharides (30,31). Stabilization energies of modified
157 TS relative to unmodified TS were given. Molecular graphics and analysis were performed using BIOVIA
158 Discovery Studio Visualizer (Dassault Systèmes, 2020).

159

160 **Statistical analyses**

161 All experiments are at least 3 independent replicates unless stated otherwise. Statistical analyses were
162 performed with Prism 8. Further details are in **Supplemental Experimental Procedures**.

163

164 **Data availability**

165 Microarray GSE104645 data are available in the Gene Expression Omnibus (GEO) repository,
166 <https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE104645>.

167

168 **Results**

169 ***O*-GlcNAcylation potentiates CRC sensitivity to 5-FU chemotherapy *in vivo***

170 To investigate the effect of *O*-GlcNAcylation on CRC progression and response to 5-FU *in vivo*, we induced CRC
171 in C57BL/6J OlaHsd mice using azoxymethane (AOM)/ Dextran Sulfate Sodium (DSS) combination
172 **(Supplemental Fig. S1a)** that was shown to mimic a wide variety of tumors of all CRC consensus molecular
173 subgroups (32). The induction of tumors was checked by colonoscopy 10 weeks after AOM injection

174 (Supplemental Fig. S1a and b). Vehicle, 5-FU and/or Thiamet-G, a pharmacological inhibitor of OGA, was then
175 delivered continuously for 13 days. Before sacrifice, tumor number and grade were analyzed and scored by
176 colonoscopy. Consistent with the literature, immunoblotting and immunohistochemistry showed
177 hyper-*O*-GlcNAcylation of proteins in CRC tumors (+AOM) compared to healthy tissues without affecting OGT
178 levels (Supplemental Fig. S1c and d). An elevated *O*-GlcNAcylation level was accompanied by an increase in TS
179 amount (Supplemental Fig. S1c and d). In AOM/DSS treated mice, in comparison to control treatment, tumor
180 number was reduced upon Thiamet-G or 5-FU treatment, and in mice that received both drugs (Fig. 1a and b).
181 All treatments reduced low-grade 1 tumors but only combined treatment reduced those of high-grade 4 and 5
182 tumors (Fig. 1c). Thiamet-G and 5-FU alone or in combination decreased the percentage of mice with grade 5
183 tumors and increased those with grade 3 and 4 (Fig.1d). The percentage of grade 3 tumors was higher in
184 co-treatment condition than in Thiamet-G or 5-FU treatment alone, suggesting that the combination of 5-FU
185 and Thiamet-G further delays the progression of the disease. In AOM/DSS treated mice, Thiamet-G treatment
186 triggered an increase of global *O*-GlcNAcylation in CRC tumors which was associated with a decrease in OGT
187 levels (Fig. 1e) suggesting a compensatory mechanism as previously reported (33). Elevation of
188 *O*-GlcNAcylation was also accompanied with a trend of increased TS levels (Fig. 1e and f). As expected (34), we
189 observed the heavy TS isoform (38 kDa) complexed with 5,10-MTHF and FdUMP confirming 5-FU incorporating
190 into CRC tissue (Fig. 1e). 5-FU treatment led to a decrease of global *O*-GlcNAcylation without affecting OGT
191 levels (Fig. 1e). Interestingly, Thiamet-G induced increase in *O*-GlcNAcylation returned close to control levels
192 upon 5-FU co-treatment in CRC tumors (Fig. 1e) and more precisely in the epithelium (Fig. 1f). TS levels were
193 further increased in co-treatment condition compared to the 5-FU condition alone (Fig. 1e and f). Thus, *in vivo*
194 data suggest a crosstalk between *O*-GlcNAcylation and 5-FU metabolism, and a sensitizing effect of
195 *O*-GlcNAcylation to 5-FU cytotoxicity possibly by increasing TS protein levels. Finally, we examined the
196 relevance of this data in the human physiopathology from the microarray dataset GSE104645. We analyzed the
197 *OGT* and *TYMS* mRNA expression profiles from primary non-treated tumors of metastatic CRC patients
198 subsequently treated with first line 5-FU-based chemotherapy (Fig. 1g and Supplemental Table S2).

199 Interestingly, while *TYMS* expression was not correlated to chemotherapy response in this cohort, *OGT* mRNA
200 level was significantly higher in responders compared to non-responder patients (**Fig. 1g**) suggesting that high
201 expression of *OGT* and the consequent higher *O*-GlcNAcylation could modulate 5-FU sensitivity.

202

203 **5-FU decreases cellular *O*-GlcNAcylation *in vitro***

204 To decipher the relationship between *O*-GlcNAcylation, TS and cellular response to 5-FU, we first compared
205 *OGT*, *O*-GlcNAcylation and TS levels in CCD 841 CoN non-cancerous and cancerous cell lines. As expected, *OGT*,
206 *O*-GlcNAcylation and TS levels were increased in cancer cells compared to non-cancerous ones (**Fig. 2a**). While
207 *OGT* and *O*-GlcNAcylation protein levels are similar between parental HT-29 and 5-FU resistant HT-29 5F31
208 cells, TS level is 10-fold higher in HT-29 5F31 compared to HT-29 cells (**Fig. 2a**) due to amplification of *TYMS*
209 gene (23). Also, and as expected, HT-29 5F31 showed an IC₅₀ to 5-FU of three log higher than its HT29
210 counterpart and CCD 841 CoN (**Fig. 2b**). CCD 841 CoN cells were less sensitive to S-phase dependent 5-FU
211 cytotoxicity than HT-29 cells (**Supplemental Fig. S2a and b**) most likely due to their very low growth rate.
212 Compared to parental HT-29 cells and as expected, HT-29 5F31 cells showed a strong resistance to 5-FU
213 treatment (**Fig. 2b and Supplemental Fig. S2a and b**) probably due to high TS expression (**Fig. 2a**). In all
214 subsequent experiments, we treated both cell lines with the 5-FU clinical concentration used of 6 μM, which
215 induced S-phase arrest within 24 h and apoptosis at 72 h in HT-29 cells (**Supplemental Fig. S2a and b**). We next
216 investigated the effect of 5-FU treatment on *OGT* and *O*-GlcNAcylation levels. Upon 24 h of treatment, 5-FU led
217 to decreased protein *O*-GlcNAcylation (**Fig. 2c**) concurrent with a decrease of both *OGT* mRNA (**Fig. 2d**) and
218 protein (**Fig. 2c**) levels in CCD 841 CoN and HT-29 cell lines while it had no effect on HT-29 5F31 cells. These
219 results indicate that 5-FU affects *O*-GlcNAcylation by decreasing *OGT* at a transcriptional level in non-cancerous
220 and cancerous colon cells but not in 5-FU resistant ones.

221

222 ***O*-GlcNAcylation modulates sensitivity to 5-FU and TS activity in colon cancer cells**

223 We then wondered whether *O*-GlcNAcylation could impact sensitivity to 5-FU by regulating TS. siRNA
224 downregulation of *OGT* led to a decrease of protein *O*-GlcNAcylation and TS levels (**Fig. 3a**). In CCD 841 CoN
225 cells, *OGT* knockdown drastically reduced free TS level (**Fig. 3a**) and cellular activity (**Fig. 3b**) by approximately
226 75% in untreated cells but had no effect on 5-FU treated cells. 5-FU or *OGT* downregulation did not affect cell
227 cycle progression of CCD 841 CoN cells (**Supplemental Fig. S3a**). In HT-29 cells, siOGT treatment led to a drastic
228 decrease of TS level (**Fig. 3a**) and cellular activity in control and 5-FU treatment conditions (**Fig. 3b**). In all cell
229 lines treated or not with 5-FU, *OGT* knockdown did not affect *TYMS* mRNA level (**Fig. 3c**) suggesting that the TS
230 regulation would occur at protein level. siOGT did not modulate either intrinsic TS activity (**Supplemental Fig.**
231 **S3b**) nor TS subcellular localization (**data not shown**). 5-FU treatment induced S-phase arrest and apoptosis of
232 HT-29 cells as observed by the increase of sub-G₁ phase cell population (**Supplemental Fig. S3b**) and the PARP-
233 1 cleavage (**Fig. 3a**). *OGT* knockdown in HT-29 cells led to an increase of the 5-FU IC₅₀ compared to control
234 condition (36.62 μM ± 1.68 versus 26.84 μM ± 1.22 respectively) (**Fig. 3d**) along with a reduction of apoptosis
235 by more than 30% (**Fig. 3a and Supplemental Fig. S3b**). These results suggest that reduced *O*-GlcNAcylation
236 would counteract sensitivity to 5-FU possibly by decreasing TS amount. We then hypothesized that increasing
237 TS levels would counteract siOGT effects on HT-29 5-FU sensitivity. Overexpression of 3xFLAG-TS (**Fig. 3e**)
238 rendered HT-29 cells resistant to 5-FU (**Fig. 3f**). siOGT decreased both endogenous and 3xFLAG-TS levels
239 confirming that *O*-GlcNAcylation regulates TS protein level in a transcription independent manner. Reduction
240 of sensitivity to 5-FU induced by siOGT was counteracted by the overexpression of recombinant 3xFLAG-TS
241 (**Fig. 3e and f**). In HT-29 5F31 cells overexpressing TS, siOGT did not affect neither levels nor intrinsic activity of
242 TS as expected (**Fig. 3a and b**). The cell cycle distribution of siOGT transfected HT-29 5F31 cells remained
243 unchanged in presence of 5-FU (**Supplemental Fig. S3b**). Together, our data showed that knockdown of *OGT*
244 lowered cancer cell sensitivity to 5-FU by decreasing TS levels and consequently its cellular activity in non-
245 cancerous and cancerous 5-FU sensitive cells but not in their resistant counterparts.

246

247 **TS interacts with OGT and is *O*-GlcNAcylated**

248 We next hypothesized that TS is regulated by *O*-GlcNAcylation and that GlcNAc precursors, glucose and
249 glucosamine (GlcNH₂), regulate its levels. In HT-29 cells, *O*-GlcNAcylation levels are lowered or barely
250 detectable under low glucose (LG, 5 mM) or no glucose (OG) conditions respectively (**Fig. 4a**). The reduced *O*-
251 GlcNAcylation levels could be restored by complementing OG media with GlcNH₂ (**Fig. 4a**). Conversely, high
252 glucose media (HG, 25 mM) enhanced *O*-GlcNAcylation levels compared to LG condition (**Fig. 4a**). TS levels
253 were highly decreased under glucose deprivation, and were partially restored by GlcNH₂ treatment. More
254 notably, reduced glucose levels and *OGT* knockdown led to a decrease in TS (**Fig. 4a**). These results indicate
255 that TS expression level is impacted by *O*-GlcNAcylation in a nutrient dependent manner. We then performed a
256 set of experiments to get more insight on the *O*-GlcNAcylation of TS. Co-immunoprecipitation experiments in
257 HT-29 5F31 cells that highly express TS (**Fig. 2a**) showed that *OGT* interacts with TS in both control and 5-FU
258 treated cells (**Fig. 4b**). Immunoblotting of immune purified TS from HT-29 cells lysate with a pan anti-*O*-GlcNAc
259 antibody revealed a signal that was decreased by *OGT* silencing, showing the *O*-GlcNAc modification of the
260 enzyme (**Fig. 4c**). Interestingly, while siOGT decreased total TS proteins (input), it slightly affected
261 *O*-GlcNAcylation of immunoprecipitated TS suggesting that this PTM is abundant and/or stable, and may have a
262 major impact on TS protein levels. We then performed *O*-GlcNAc mass tag experiments using a 4.4 kDa PEG to
263 monitor TS *O*-GlcNAc stoichiometry in CCD 841 CoN and HT-29 cells. In both cell lines, we observed a
264 PEGylated-*O*-GlcNAc-TS isoform with an apparent molecular weight shift of + 4-5 kDa showing that TS is the
265 recipient of one *O*-GlcNAcylation (**Fig. 4d**). In HT-29 cells, a second PEGylated-*O*-GlcNAc-TS isoform bearing 4
266 *O*-GlcNAc was also detected as shown by an apparent molecular weight shift of + 17-18 kDa. Interestingly,
267 PEG-*O*-GlcNAc-TS underwent an extra shift upon 5-FU treatment revealing that the complexed TS isoform is
268 also *O*-GlcNAcyated in both cell lines. We also noted that TS *O*-GlcNAcylation levels were higher in cancer cells
269 (27% of total TS) compared to non-cancerous cells (9%) and that 5-FU did not significantly affect the TS
270 *O*-GlcNAc stoichiometry (**Fig. 4d**). We next mapped TS PTMs by HCD-MS/MS in HT-29 and HT-29 5F31 cells
271 treated with Thiamet-G to stabilize *O*-GlcNAcylation. We identified an *O*-GlcNAcyated peptide covering the
272 T234, T241 and T251 (**Fig. 4e**). We precised the *O*-GlcNAc position at the T251 residue and its role on TS

273 stabilization by transfection of the three single threonine mutated TS-FLAG (**Supplemental Fig S4**). Our MS data
274 also pointed out an *O*-GlcNAc site at the T306 (**Fig. 4e**) along with the previously described (35)
275 phosphorylation showing a reciprocal phosphorylation/*O*-GlcNAcylation interplay on this residue. We also
276 mapped two phosphorylated sites at S114 and T170 (**Supplemental Table S3 and Fig. S5**).

277

278 ***O*-GlcNAcylation increases TS stability by preventing its proteasomal degradation**

279 Since OGT and *O*-GlcNAcylation are known to control protein expression and stability (36), we investigated
280 whether *O*-GlcNAcylation would stabilize TS. We generated series of *O*-GlcNAcylation mutants (T251A, T306A
281 and T251A/T306A) and two phosphomimetic mutants (T306D and T251A/T306D). Level of the T251A mutant
282 was lower than the wild type about 45% (**Fig. 5a**). Neither the substitution of T306 to alanine (T306A) nor to
283 aspartic acid (T306D) affected the TS expression levels compared to the wild type. The double mutant
284 T251A/T306A exhibited a strongly decreased-level of about 75% that was restored close to wild type levels for
285 the double mutant T251A/T306D (**Fig. 5a**). This data suggests that *O*-GlcNAcylation at T251 and
286 phosphorylation/*O*-GlcNAcylation interplay at T306 control TS protein level. We then performed a time-course
287 experiment in which HT-29 cells were treated with cycloheximide with or without Thiamet-G to analyze TS
288 stability (**Fig. 5b**). Thiamet-G alone induces a slight decrease of TS expression and stability because of the high
289 TS *O*-GlcNAcylation stoichiometry (**Fig. 4c and d**). Twenty-eight hours after cycloheximide protein synthesis
290 inhibition, only 10% of TS remained detectable, while cells co-treatment with Thiamet-G exhibited about 20%
291 of remaining TS (**Fig. 5b**). This data showed that forced-enhancement of *O*-GlcNAcylation increased TS lifetime.
292 We then analyzed TS levels after treatment with siOGT with or without the proteasome inhibitor MG132 (**Fig.**
293 **5c**). Reduction of TS levels induced by *OGT* knockdown was partially restored upon MG132 treatment
294 indicating that *O*-GlcNAcylation protects TS from proteasomal degradation in an ubiquitin-independent manner
295 (37, 38) (**Fig. 5c and Supplemental Fig. S6**). Furthermore, MG132 treatment restored protein levels of T251A
296 and T251A-T306A mutants (**Fig. 5d**), reinforcing the role of *O*-GlcNAcylation of these residues in the TS
297 protection toward proteasomal degradation. Our results revealed that *O*-GlcNAcylation of TS at T251 and

298 *O*-GlcNAcylation/phosphorylation interplay at T306 regulate proteasomal degradation and stability of the
299 enzyme. To get more insight on how PTMs affect TS stability, the full human TS protein was modeled (**Fig. 6a**)
300 and empirical potential energies of stabilization (ΔE) of modified TS were calculated. *O*-GlcNAcylation at T306
301 slightly increased TS stability ($\Delta E = -5.8 \text{ kcal.mol}^{-1}$) while phosphorylation at T306 and *O*-GlcNAcylation at T251
302 similarly and strongly stabilized it ($\Delta E = -29.7$ and $-32.5 \text{ kcal.mol}^{-1}$ respectively) (**Fig. 6b**). In accordance to
303 experimental data (**Fig. 5a**), modification of both amino acids increased further this stability ($\Delta E = -42.4$
304 and $-53.2 \text{ kcal.mol}^{-1}$ for *O*-GlcNAcylation at T251 and *O*-GlcNAcylation or phosphorylation at T306 respectively).
305 Stabilization has originated from strong ionic and van der Waals interactions, and hydrogen bonds between TS
306 amino acids and phosphate or *O*-GlcNAc moiety (**Fig. 6c**). TS dimerization seems to be driven partly by
307 *O*-GlcNAcylation since glycosylation at T251 of monomer A generates hydrogen and π - σ bonds with amino
308 acids located in TS monomer B at the dimer interface (at G60, M61, E62, T251, L252 and Y213 residues
309 respectively) (**Fig. 6c**). This network of links does not seem to be generated by the other structures proposed.

310

311 Discussion

312 5-FU alone or combined with other drugs is one of the most frequently used chemotherapy for CRC treatment.
313 Nonetheless, the outcome is often sub-optimal because of resistance to 5-FU treatment. Since TS is a key
314 target of 5-FU, the possible mechanism of 5-FU response in CRC is likely to involve this enzyme. Proteomic and
315 systematic approaches identified TS as an *O*-GlcNAcylated protein (8, 9) but the role of this PTM on the enzyme
316 remained unknown. Glycosylation alterations play an important role in CRC response to several therapies (39).
317 To test this conjecture, we first investigated TS expression and *O*-GlcNAcylation levels in CRC tissues. Consistent
318 with previously published data, *O*-GlcNAcylation levels are increased in colorectal tumors compared to normal
319 tissues (40–42). AOM/DSS treatment induces tumorigenesis that activates glucose and glutamine consumption
320 to support cell proliferation. Increased nutrient availability, subsequent flux through Hexosamine Biosynthetic
321 Pathway (HBP) and/or OGT activity lead to hyper *O*-GlcNAcylation in tumor tissues without necessary alteration
322 in OGT abundance (43,44). TS levels are also increased in tumors highlighting the significant need for dTMP

323 synthesis to support cell proliferation (45). We showed that nutrients affect TS levels by regulating
324 O-GlcNAcylation and that TS O-GlcNAcylation is increased in colon cancer cells compared to non-cancerous
325 ones. More interestingly, we present evidence that enhanced TS levels by O-GlcNAcylation sensitizes CRC to
326 5-FU cytotoxicity and decipher the underlying molecular mechanism. Elevation of O-GlcNAcylation *in vivo* by
327 Thiamet-G correlated with increased TS levels and potentiated 5-FU cytotoxic effect in a murine model of CRC
328 as shown by number and grade tumor analyses. We showed that Thiamet-G alone reduces CRC progression. It
329 was previously shown that increased O-GlcNAcylation by heterozygote knockout of *Meningioma Expressed*
330 *Antigen 5 (MGEA5)* encoding OGA attenuates tumorigenesis and enhances survival in sporadic *Adenomatous*
331 *Polyposis coli (APC)^{min/+}* CRC mice model (46). Consistently with our and others' data showing that knockdown
332 of *OGT* or *MGEA* reduces cell growth (45, 46), these studies highlight the pivotal role of O-GlcNAcylation
333 homeostasis in carcinogenesis and tumor growth. Data mining of CRC patients revealed that *OGT* expression is
334 positively correlated with the 5-FU-based chemotherapy response. Consistently, downregulation of *OGT* and
335 O-GlcNAcylation decreased *in vitro* 5-FU cytotoxic effect by regulating both TS stability and levels, and
336 subsequent cellular enzyme activity. *TYMS* gene overexpression is a currently thought to be a biomarker of
337 5-FU resistance in CRC (3). However, the variability of genetic background based on *TYMS* gene amplification or
338 polymorphisms (49), transcription and translation induced by 5-FU (49–51), ratio between the FdUMP-
339 complexed and the free form of TS (52) and final TS protein expression (53) can also modulate the sensitivity to
340 the drug. The patient TS protein expression might result from several regulatory changes at many levels such as
341 transcription, post-transcriptional regulation, translation, and post-translational regulation (54). Distinguishing
342 between mRNA overexpression and enzyme post-translational stabilization as a regulation mechanism of TS
343 proteins has implications with regard to how cancer cells may respond to 5-FU therapy. Although cells with low
344 TS levels might theoretically be more sensitive to 5-FU, the subsequent low proliferation rate prevents
345 induction of DNA damage and 5-FU toxicity as we show in non-cancerous CCD 841 CoN cells. In parallel, HT-29
346 5F31 cancer cells which exhibit high TS level due to *TYMS* gene amplification are also resistant to 5-FU. Thus,
347 we suggest that the regulation of 5-FU response by O-GlcNAcylation is finely tuned and depends on a proper

348 amount of TS proteins. Moreover, *O*-GlcNAcylation strongly stabilizes TS by forming novel intra- and inter-
349 monomer interactions (hydrogen and π - σ bonds). Potential PTM induced-structure modifications of TS could
350 also increase its affinity for FdUMP metabolites and thus enhancing TS inhibition.

351 We also reveal a reciprocal effect of 5-FU on *O*-GlcNAcylation levels *in vitro* and *in vivo*. 5-FU treatment
352 affected *O*-GlcNAcylation by decreasing OGT at both protein and mRNA levels in non-cancerous and 5-FU
353 sensitive cancerous colon cell lines. In murine CRC tumors, OGT levels were steady under 5-FU treatment. This
354 difference could be due to that OGT levels were analyzed after two weeks of systemic 5-FU treatment in mice
355 allowing possible return to equilibrium while cell lines received 72 hours acute treatment. Additionally, the 5-
356 FU-induced decrease of protein *O*-GlcNAcylation in murine CRC tissues could be related to decreased OGT
357 activity rather than to decreased OGT level. Since OGT activity is inhibited by high UDP, UTP and UDP-GlcNAc
358 concentrations (55), 5-FU metabolites may inhibit OGT by producing fluorinated derivatives of uridine
359 compounds (56). We demonstrate that 5-FU decreases *OGT* expression and cellular *O*-GlcNAcylation without
360 affecting *O*-GlcNAcylation of TS suggesting that this PTM is abundant and/or stable. Under 5-FU treatment, the
361 *O*-GlcNAcylation stabilizes TS whose major FdUMP-complexed isoform is inhibited leading to cell cycle arrest
362 and apoptosis. Together, our study highlights a crosstalk between *O*-GlcNAcylation and 5-FU metabolism *in*
363 *vitro* and *in vivo* which is at the benefit of stabilizing the 5-FU target TS by *O*-GlcNAcylation, hence enhancing
364 the sensitivity to 5-FU.

365 We show here that TS *O*-GlcNAcylation at T251 and T306 stabilizes the enzyme by preventing its proteasomal
366 degradation in ubiquitin-independent pathway. TS proteasomal degradation is controlled by two degron
367 sequences M1-R42 and F276-V313 located respectively in the N- (N-ter) and C-terminal (C-ter) regions (57). We
368 report for the first time an *O*-GlcNAc modification at T251, a key residue located in the sixth β -strand at the
369 dimer interface of TS (58). We show that the T251A mutation decreases TS stability. Recently, Pozzi et al.
370 (2019) showed that the substitution of Q62N at the dimer interface destabilizes TS homodimer by inducing a
371 slight aperture of the TS dimer (59). In a similar manner, the T251A mutation could decrease stability of TS by
372 modifying *O*-GlcNAc induced-interactions and reducing its dimerization. Interestingly, T306 is located within

373 the C-ter cryptic degnon whose activity is regulated by the N-ter degnon (57). The sole mutation T306A does not
374 affect TS stability in accordance with the observation that deletion of P305-I307 sequence comprising the T306
375 residue does not affect stability of TS (38). Conversely, in combination with T251A mutation the absence of
376 modification at T306 enhances TS degradation. This result is reinforced by our data showing that the T306D
377 phosphomimetic mutation is able to restore stability of T251A mutants suggesting that phosphorylation of this
378 site would inhibit the TS degnon activity. Remarkably, the P305-I307 sequence of TS is similar to the
379 P375-T-L377 tripeptide motif that dictates the ubiquitin-independent degradation of c-Fos protein.
380 Degradation of c-Fos depends on the activity of N-ter and C-ter degons that are regulated by phosphorylation
381 and possibly by heterodimerization of c-Fos with various partners, the best known being the Jun family
382 members (60). TS degnon activity could also be regulated by PTMs and dimerization. Here, we report that the
383 T251A mutation decreases TS stability that can be restored by phosphorylation at T306, indicating that TS
384 proteasomal degradation driven by hypo-*O*-GlcNAcylation at T251 depends on
385 *O*-GlcNAcylation/phosphorylation status at T306 of the C-ter degnon. Relative empirical potential stabilization
386 energy calculations indicate that both *O*-GlcNAcylation and phosphorylation at T306 increases stability of T251
387 *O*-GlcNAcylated TS.

388 In agreement with our findings, the beneficial effect of *O*-GlcNAcylation in response to chemotherapy has also
389 been documented for cisplatin in ovarian cancer (16,17), for bortezomib in mantle cell lymphoma (19) and for
390 TRAIL therapy in various cancers (14). Thiamet-G is the most widely used OGA inhibitor *in vitro* and *in vivo* as it
391 exhibits excellent stability and selectivity (61). Two others selective OGA inhibitors (MK-8719 and ASN120290)
392 have been recently included in the orphan drug designation program of the US Food and Drug Administration
393 for the safe and effective treatment of the progressive supranuclear palsy, a neurodegenerative tauopathy. Our
394 study shows that *O*-GlcNAc homeostasis-TS axis plays an important role in mediating 5-FU sensitivity in CRC,
395 hence therapeutic combination strategy of 5-FU with an OGA inhibitor would be beneficial for CRC patients.
396 More broadly, several existing potent inhibitors of HBP enzymes, OGT and OGA have shown interests in

397 anti-cancer therapies (62). Therefore, targeting *O*-GlcNAcylation in combination with chemotherapy in
398 pre-clinical models should be explored further.

399 For summarize, in this paper, we show that a combined treatment of 5-FU with Thiamet-G has a synergistic
400 inhibitory effect on CRC progression. We evidence a crosstalk between *O*-GlcNAcylation, TS and 5-FU
401 cytotoxicity. We report that *O*-GlcNAcylation stabilizes the human TS and sensitizes to 5-FU effect. We in-depth
402 document the *O*-GlcNAc T251 site-specific function along with its interplay with phospho/*O*-GlcNAc T306 on TS
403 structure and stability. Since, resistance to 5-FU is the major obstacle to therapy success, comprehension of
404 molecular mechanism of TS regulation that potentiates 5-FU effectiveness is of high importance to open a new
405 paradigm of cancer therapeutic options.

406

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417

418 **Author contributions**

419 NV designed, performed and analyzed *in vitro* and *in vivo* experiment data and co-wrote the manuscript. SH
420 performed plasmid constructions and PEG synthesis and cowrote the manuscript. AD contributed to the *in vivo*
421 experiments. JKC contributed to the *in vivo* experiment design and the reviewing of the manuscript. JT

422 contributed to the *in vivo* experiments. MD performed mice colonoscopy and contributed to the IHC
423 experiments. AP performed mass spectrometry analyzes. GV performed TS structure modeling in silico analysis.
424 CS performed microscopy acquisition of fluorescence images of immunocytochemistry experiments. TL
425 contributed to discussions and reviewed the manuscript. VD contributed to the work design, the experiments,
426 the data analysis and the reviewing of the manuscript. IEB supervised and conceptualized the research,
427 contributed to the experiments and data analyzes, and co-wrote the manuscript. All authors read and
428 approved the manuscript.

429

430

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435

436 **Competing Interests**

437 No potential conflicts of interest were disclosed.

438

439 **References**

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602

603 **Figure legends**

604 **Fig. 1. O-GlcNAcylation sensitizes CRC to 5-FU chemotherapy *in vivo*.** a-f Colorectal tumors were induced by
605 AOM/DSS and continuously treated with Thiamet-G (90 mg/kg/d) and/or 5-FU (12,5 mg/kg/d) during 13 days.
606 Colonoscopy was performed before sacrifice. **a** Representative endoscopic images of tumors. **b** Quantification
607 of tumor number as observed by endoscopy. Data is median \pm SEM, $n \geq 6$. **c** Quantification of tumor number by
608 grade as observed and scored by endoscopy. Data is mean \pm SEM, $n \geq 6$. **d** Histogram quantification of
609 percentage of mice with the tumor indicated highest grade. **e** Levels of OGT, O-GlcNAcylation and TS in tumors.
610 Samples were immunoblotted with indicated antibodies (*left panel*). Densitometric analysis (*right panel*). Data
611 is mean \pm SEM, $n \geq 6$. **f** Immunohistochemistry of O-GlcNAcylation and TS in tumors. Samples were stained with
612 indicated antibodies. Scale bars = 50 μ m (*left panel*). Staining intensity analysis in epithelial cells and scoring
613 (*right panel*). Data is mean \pm SEM, $n \geq 3$. **a-f** * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA test. **g**
614 Expression levels of OGT and TYMS in responder and non-responder metastatic CRC patients treated with first
615 line 5-FU-based chemotherapy from microarray dataset GSE104645. Data was normalized by the percentile
616 shift (all samples were normalized to the signal value of 75th percentile of all of probes on the microarray) and
617 the scaling (all genes were normalized to the median of all samples). Data is median \pm SEM; ** $P < 0.01$;
618 Mann-Whitney test.

619

620 **Fig. 2. 5-FU decreases OGT and O-GlcNAcylation levels in non-cancerous and cancerous cells.** **a** OGT,
621 O-GlcNAcylation and TS protein levels in CCD 841 CoN, HT-29, and HT-29 5F31 cells. Samples were
622 immunoblotted with indicated antibodies (*left panel*). Densitometric analysis (*right panel*). Data is mean \pm SD,
623 $n=3$. **b** 5-FU IC₅₀ values for CCD 841 CoN, HT-29 and HT-29 5F31 cells as determined by the MTS assay. Cells
624 were treated 48 h by increasing concentrations of 5-FU ranging from 0 to 20 μ M for CCD 841 CoN and HT-29
625 cells, and from 0 to 50 mM for HT-29 5F31 cells. Data is mean \pm SD, $n=3$. **c** Effect of 5-FU on OGT,
626 O-GlcNAcylation and TS protein levels. Cells were treated with or without 5-FU (6 μ M) for 0, 24 h, 48 h or 72 h.
627 Samples were immunoblotted with indicated antibodies (*top panel*). Densitometric analysis (*bottom panel*).

628 Data is mean \pm SD, n=3. **a and c** * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA test. **d** Effect of 5-FU on
629 OGT expression. Gene expression levels of OGT were determined by RT-qPCR in cells treated with or without 5-
630 FU (6 μ M) for 72 h. Data is expressed as relative expression. ** $P < 0.01$, *** $P < 0.001$, unpaired Student's *t*
631 test.

632

633 **Fig. 3. Knockdown of OGT reduces 5-FU sensitivity of cancer cells by decreasing TS target levels. a, b and d**

634 Cells were transfected with control or OGT siRNA (siCtrl or siOGT; 10 nM). Then, 24 hours later, cells were
635 co-treated with or without 5-FU (6 μ M) for 72 h. **a** Effects of siOGT and 5-FU treatments on OGT,
636 O-GlcNAcylation, PARP-1 and TS levels were evaluated by WB (*left panel*). Densitometric analysis (*right panels*).
637 Data is mean \pm SD, n=3. **b** Effect of siOGT and 5-FU treatments on cellular TS activity was analyzed by
638 radioactive tritium-release assay. Data is mean \pm SD, n=3. **c** Effect of siOGT and 5-FU treatment on TS
639 transcriptional levels. Gene expression level of *TYMS* was determined by RT-qPCR and data are represented as
640 relative expression. Data is mean \pm SD, n=3. **d** Effect of siOGT on 5-FU IC₅₀ was analyzed by MTS assay. Cells
641 were transfected with control or OGT siRNA (siCtrl or siOGT; 10 nM) for 24 h and then co-treated or not for 72
642 h with increasing concentrations of 5-FU ranging from 0 to 80 μ M for CCD 841 CoN and HT-29 cells and from 0
643 to 50 mM for HT-29 5F31 cells. Dose response curve to 5-FU treatment (*left panel*) and 5-FU IC₅₀ value
644 comparisons (*right panel*)

645 Data is mean \pm SD, n=3. ** $P < 0.01$, unpaired Student's *t* test. **e-f** Effect of TS overexpression on
646 siOGT-regulated apoptosis in 5-FU sensitive HT-29 cells. HT-29 cells were first transfected with indicated siRNA.
647 After 24 h, cells were transfected with the indicated constructs and treated with 5-FU (6 μ M) for 72 h prior to
648 lysis. **e** OGT, O-GlcNAcylation, TS-3xFLAG and TS WB. **f** SubG₁ apoptotic cell population was analyzed by FACS
649 after propidium iodide incorporation. Data is mean \pm SD, n=4. **a, b, d and f** * $P < 0.05$, ** $P < 0.01$, *** $P <$
650 0.001, one-way ANOVA test.

651

652 **Fig. 4. OGT interacts with and O-GlcNAcyates TS.** **a** O-GlcNAcylation regulates TS protein levels. Prior to
653 conducting the experiment, HT-29 cells were grown in low glucose (LG, 5 mM) medium for 60 h, then in
654 medium without glucose (OG) supplemented with or without glucosamine (GlcNH₂, 5 mM) or LG or high
655 glucose (HG, 25 mM) medium for 12 h. In transfection experiment cells were transfected in LG medium with
656 control or *OGT* siRNA (siCtrl or siOGT; 10 nM) and, 24 h later, grown in HG medium during the last 48 hours.
657 Samples were immunoblotted with indicated antibodies (*left panel*). Densitometric analysis (*right panel*). Data
658 is mean ± SD, n=3; * *P* < 0.05, *** *P* < 0.001, one-way ANOVA test. **b-d** Cells were treated with or without 5-FU
659 (6 μM) for 48 h. **b** OGT interacts with TS. TS and OGT were co-immunoprecipitated from HT-29 5F31 lysates and
660 samples were immunoblotted with indicated antibodies. **c** OGT O-GlcNAcyates TS. HT-29 cells were
661 transfected with siCtrl or siOGT (10 nM) and, 24 h later, incubated with or without 5-FU (6 μM) for 72 h. TS was
662 immunoprecipitated from HT-29 cell lysates and samples were immunoblotted with indicated antibodies. **d**
663 O-GlcNAc stoichiometry of TS. CCD 841 CoN and HT-29 cell lysates were O-GlcNAc mass tagged by
664 click-chemistry using a PEG tag of 4,4 kDa. Samples were immunoblotted for TS (*top left panel*). Densitometric
665 curves are shown in *bottom left panel* and densitometric analyses are presented in *top right panel*. Data is
666 mean ± SD, n=2. * *P* < 0.05, one-way ANOVA test. **e** TS is O-GlcNAcyated at T251 and T306. HT-29 and HT-29
667 5F31 cells were treated with Thiamet-G (2 μM) for 48 h. HCD-MS/MS spectrum of peptides covering the T251
668 and T306 O-GlcNAcyated sites of TS. The modification sites on TS are indicated in red in the peptide sequence.
669

670 **Fig. 5. O-GlcNAcylation at T251 and T306 increases TS stability by preventing its proteasomal degradation.** **a**
671 Expression levels of O-GlcNAc-TS mutants. HT-29 cells were transfected with empty pcDNA3.1 (mock),
672 pcDNA3.1-3xFLAG-TYMS or the indicated mutant (1 μg) for 48 h. TS-FLAG proteins were detected by WB with
673 the anti-FLAG antibody (*top panel*). Densitometric analysis (*bottom panel*). Data is mean ± SD, n=4. **b**
674 O-GlcNAcylation increases TS stability. HT-29 cells were treated or not with Thiamet-G (2 μM) and/or
675 cycloheximide (90 μg/mL) for 0, 20 h, 24 h, 28 h or 32 h. Samples were immunoblotted with indicated
676 antibodies (*left panel*). Densitometric analysis (*right panel*). Data is mean ± SD, n=3. **c** OGT knockdown induced-

677 TS decrease is dependent on proteasomal degradation. HT-29 cells were transfected with control or *OGT* siRNA
678 (siCtrl or siOGT, 10 μ M) and then, 84 h later, treated or not with MG132 (10 μ M) for 12 h prior lysis. Samples
679 were immunoblotted with indicated antibodies (*left panel*). Densitometric analysis (*right panel*). Data is mean \pm
680 SD, n=3. **d** Expression level of TS-O-GlcNAc mutants under proteasome inhibition. HT-29 cells were transfected
681 with the indicated construct and then, treated or not after 36 h with MG132 for 12 h prior lysis. Samples were
682 immunoblotted with indicated antibodies (*left panel*). Densitometric analysis (*right panel*). Data is mean \pm SD,
683 n=3. **a and d** * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA test. **b-c** * $P < 0.05$, unpaired Student's *t*
684 test.

685

686 **Fig. 6. O-GlcNAcylation at T251 and T306 increases TS dimer stabilization by generating intra- and**
687 **inter-monomer interactions.** **a** 3D modeling of human TS dimer structure. TS dimer crystal structure (green,
688 PDB: IHZW) was completed with reconstituted M1-G29 (purple) and I307-V313 (red) sequences. **b** Empirical
689 potential energies of stabilization of TS dimer whose monomer A is modified at T251 and T306 residues relative
690 to unmodified TS dimer. **c** 2D view of interactions of GlcNAc or phosphate moiety at T251 and T306 residues of
691 the monomer A with surrounding amino acids of monomers A and B.

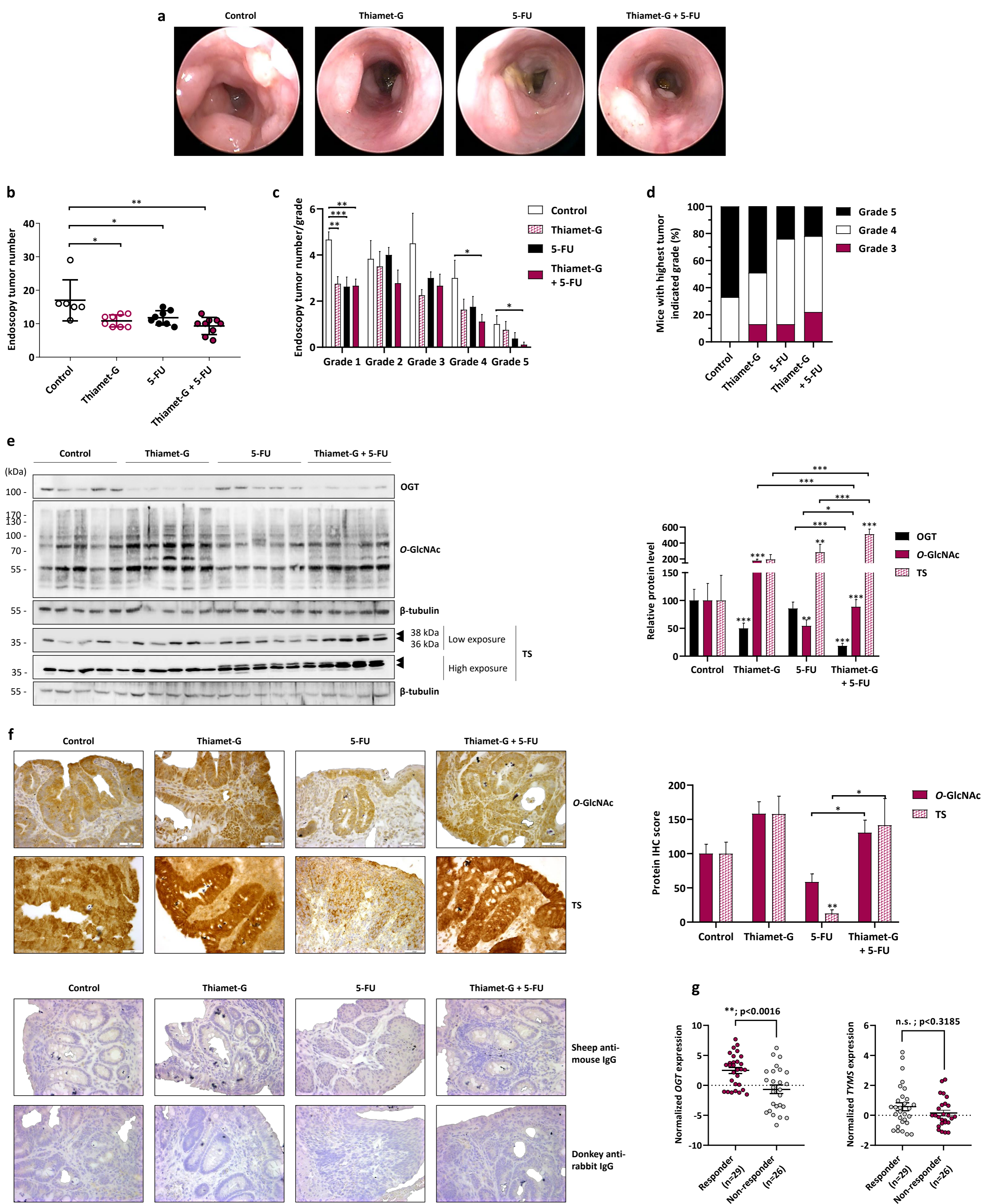


Figure 1
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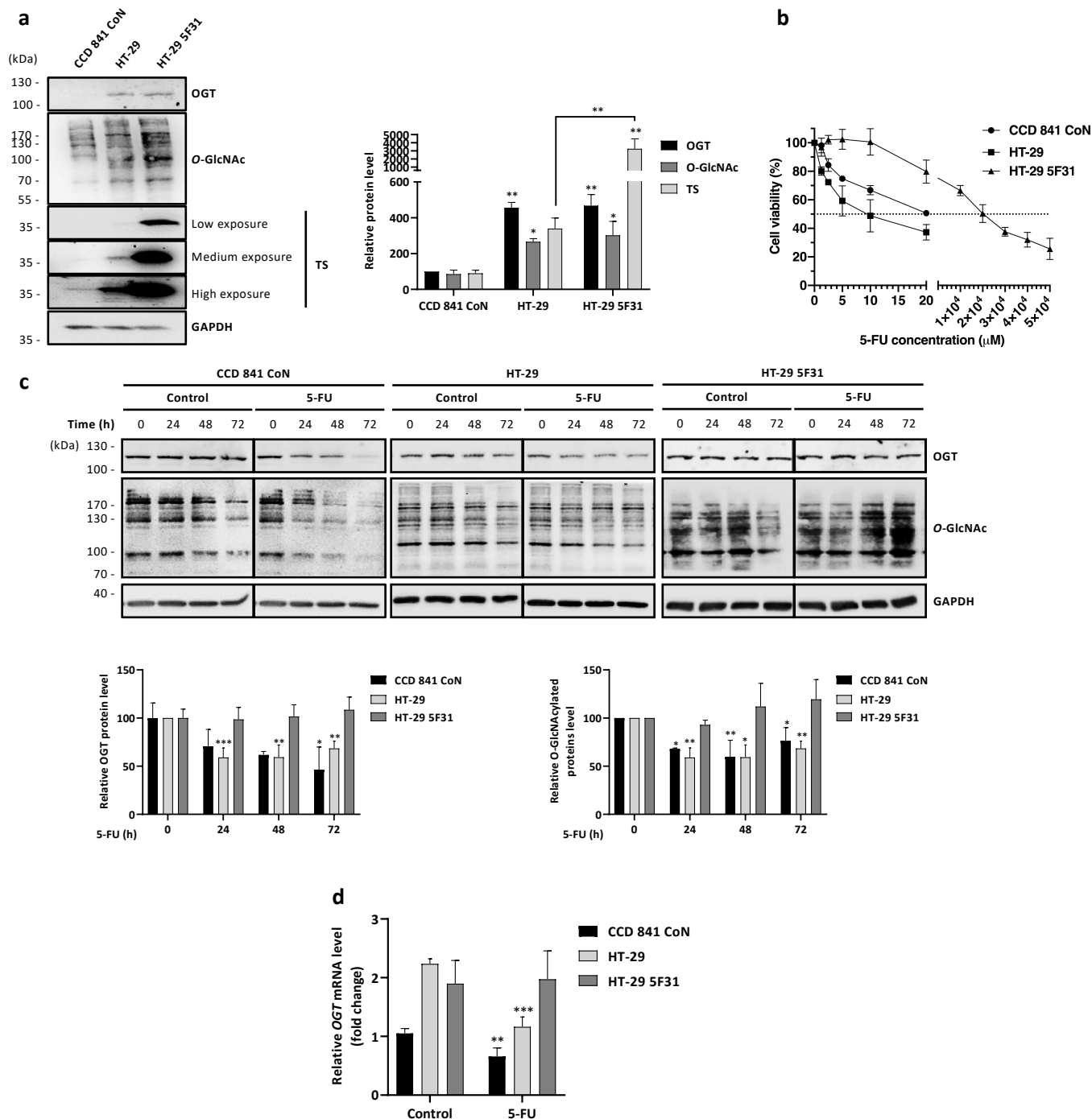


Figure 2
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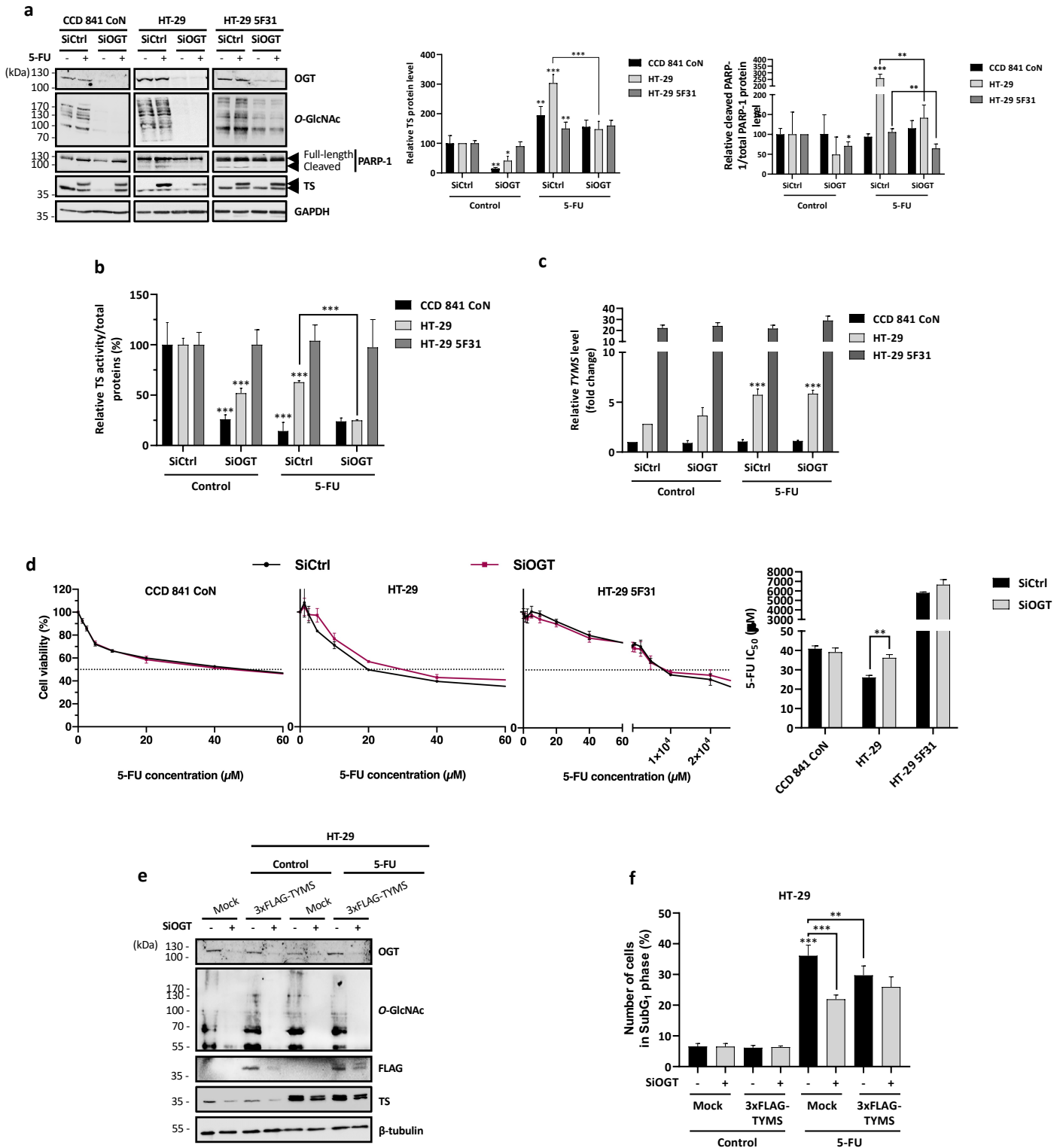


Figure 3
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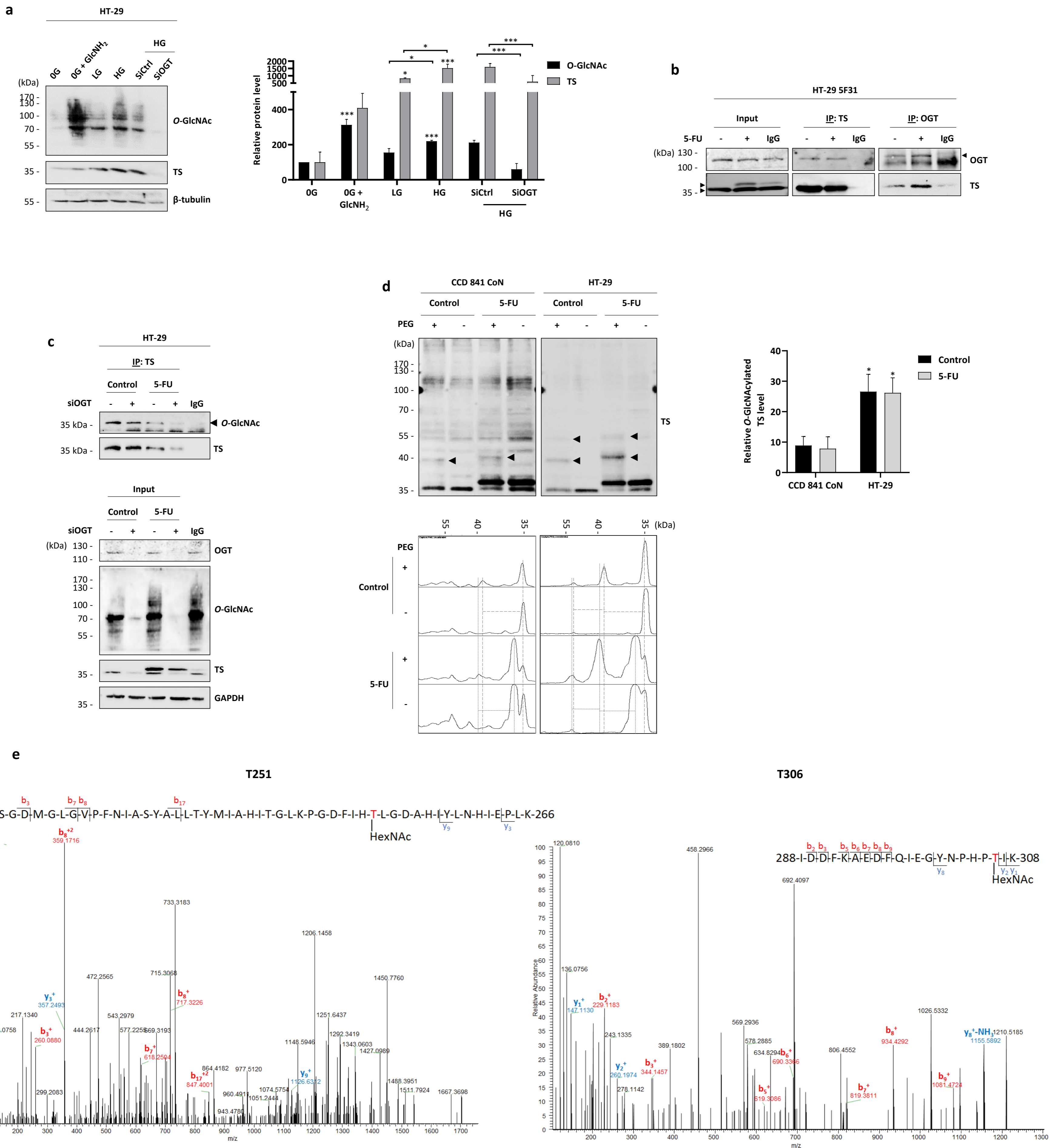


Figure 4
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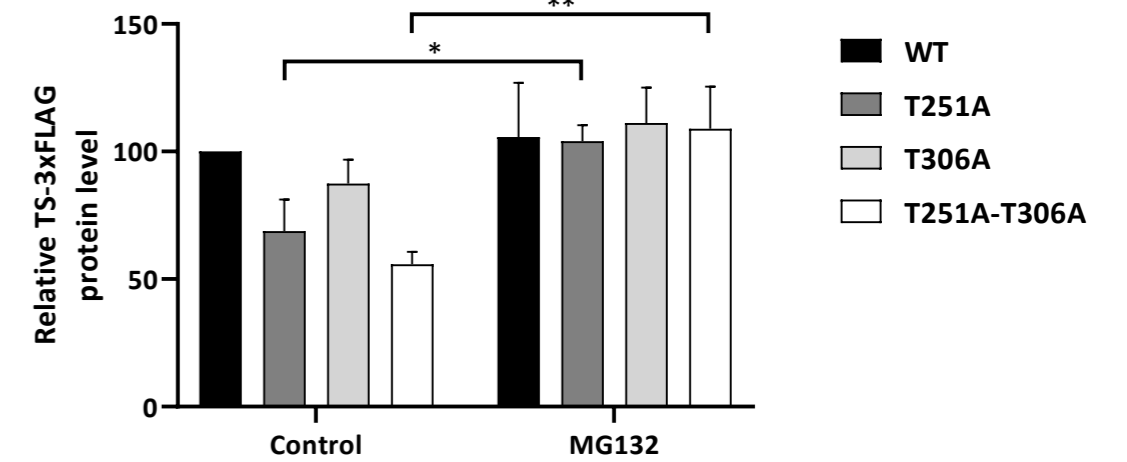
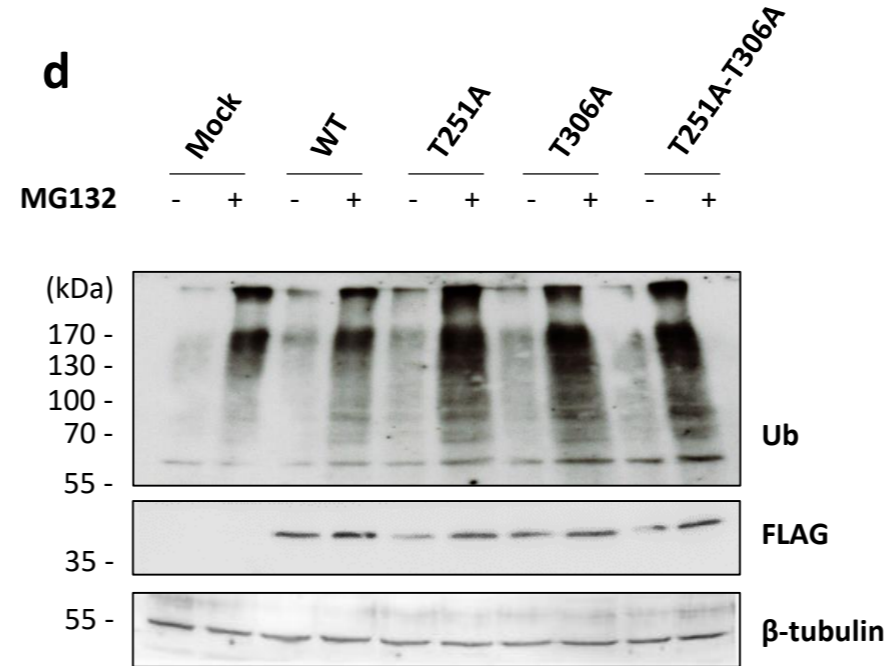
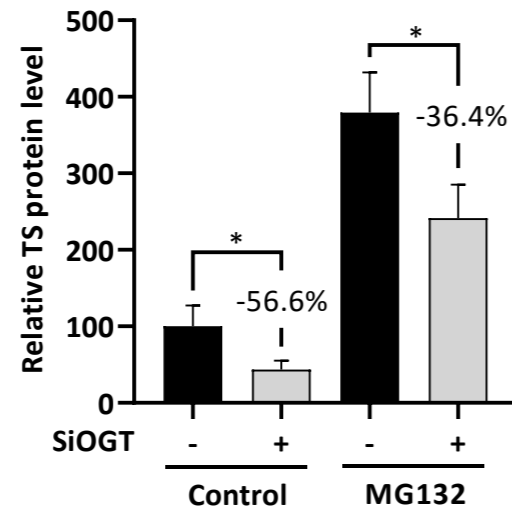
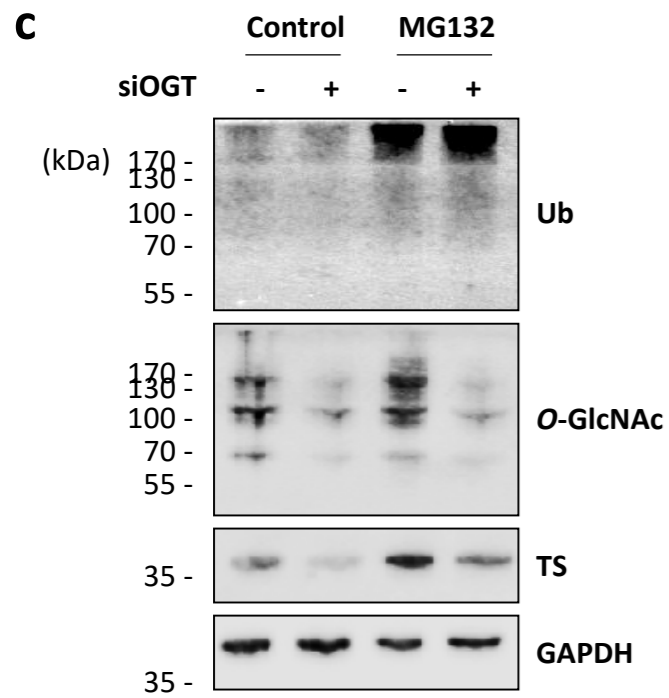
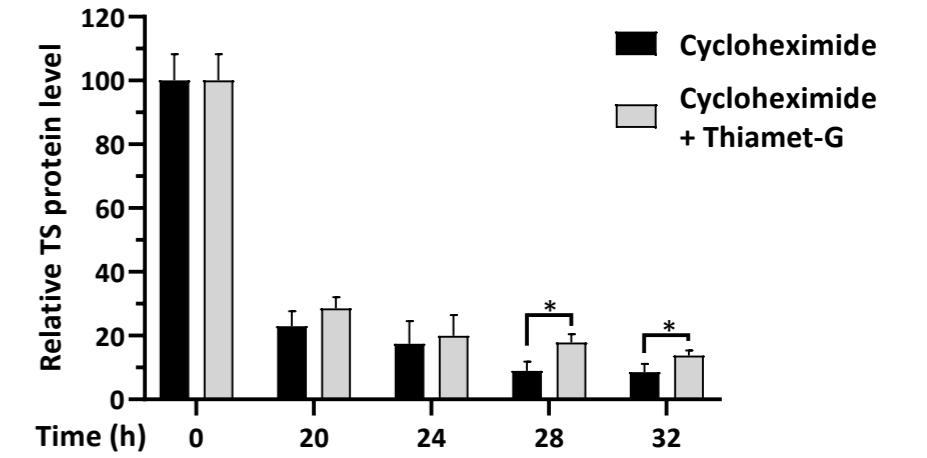
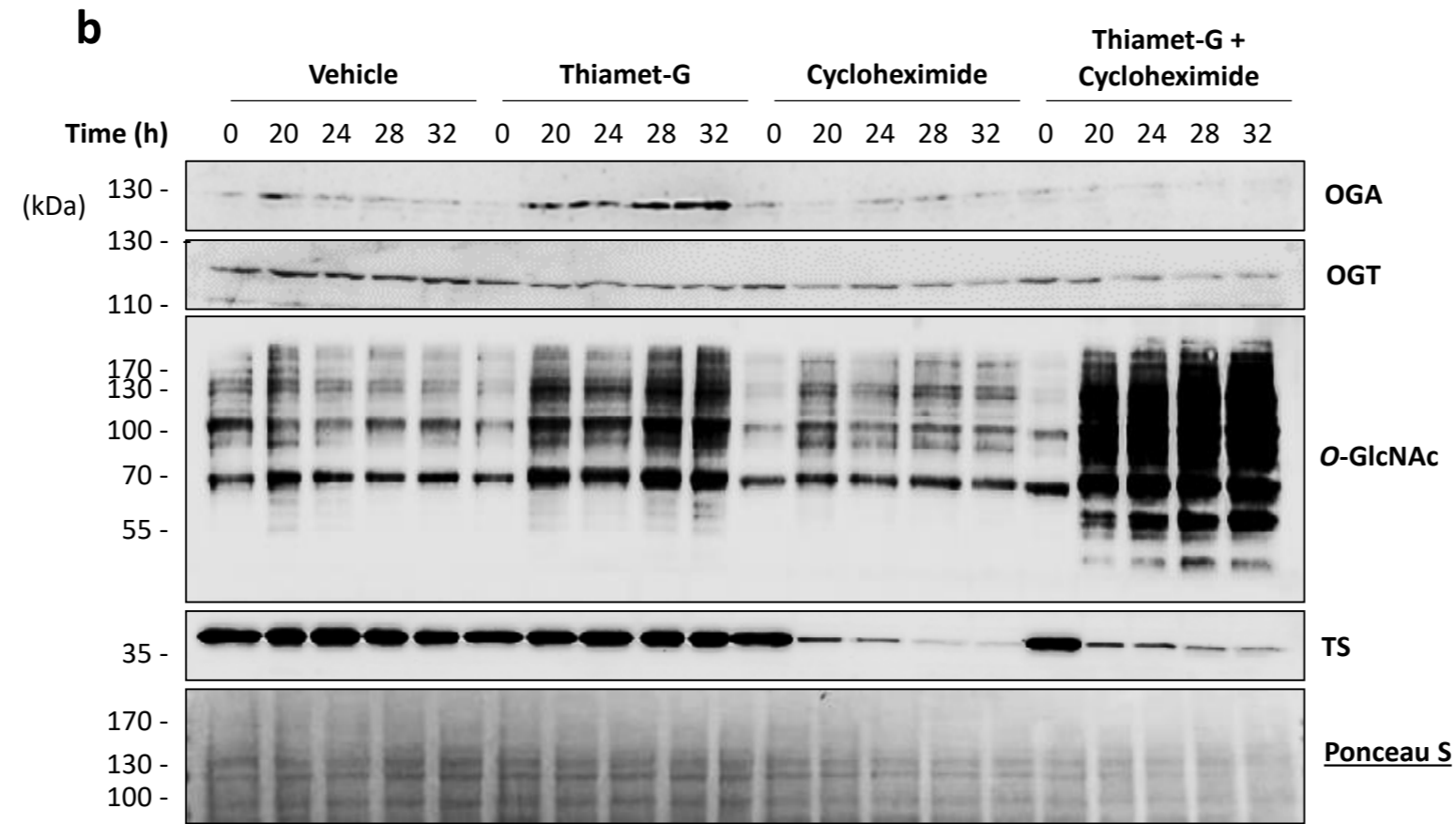
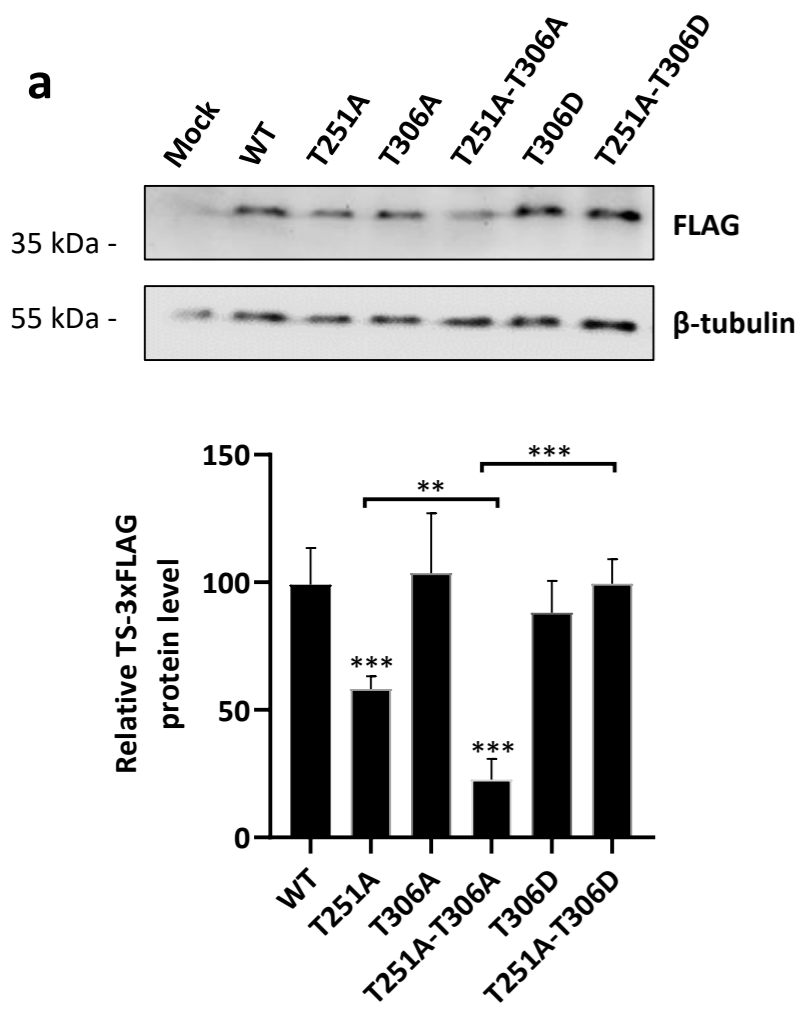


Figure 5
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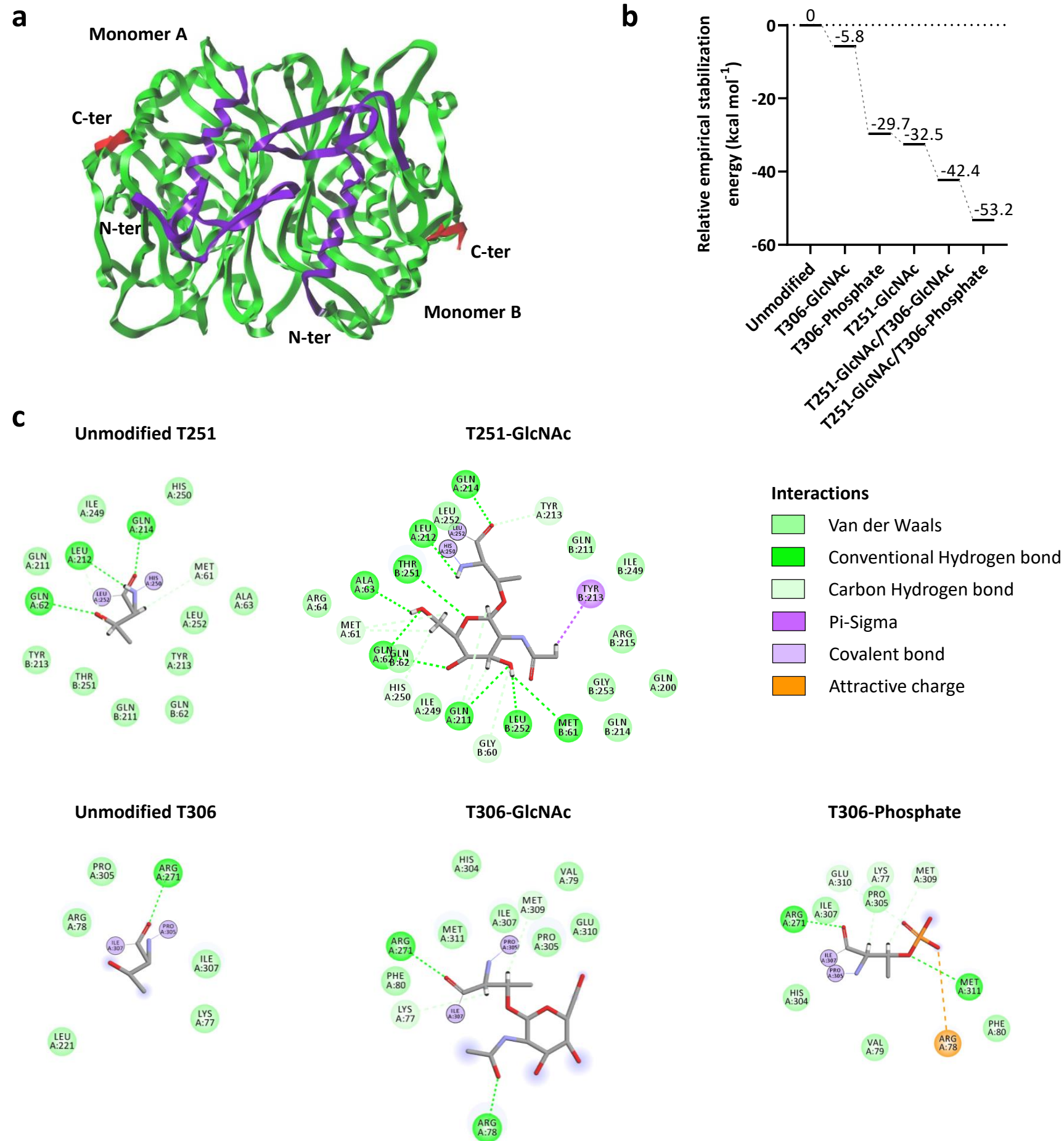


Figure 6
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