The anaphase promoting complex/cyclosome (APC/C) orchestrates various aspects of the eukaryotic cell cycle. One of its co-activators, Cdh1, is subject to myriad post-translational modifications, such as phosphorylation and ubiquitination. Herein we identify the O-linked N-acetylglucosamine (O-GlcNAc) modification that occurs on Cdh1. Cdh1 is O-GlcNAcylated in cultured cells and mouse brain extracts. Mass spectrometry identifies an O-GlcNAcylated peptide that neighbors a known phosphorylation site. Cell synchronization and mutation studies reveal that O-GlcNAcylation in regulating Cdh1 may antagonize its phosphorylation. Our results thus reveal a pivotal role of O-GlcNAcylation in regulating APC/C activity.

O-LINKED N-ACETYLGLUCOSAMINE (O-GLcNAc) MODIFICATION IS THE ADDITION OF β-N-ACETYLGLUCOSAMINE TO SERINE OR THREONINE RESIDUES OF NUCLEAR AND CYTOPLASMIC PROTEINS. O-GlcNAcylation is catalyzed by the O-GlcNAc transferase (OGT), whereas the reverse reaction is catalyzed by O-GlcNAcase (OGA). Intriguingly, the human genome contains only one OGT and only one OGA enzyme. Therefore, the OGT/OGA pair accounts for all GlcNAcylation reactions in humans. Since its discovery in the early 1980s, O-GlcNAcylation is widespread and has been identified on thousands of proteins involved in epigenetics, transcription, translation, nuclear transport, mitochondrial bioenergetics, vesicle transport, metabolism, signaling, and cellular structures. These proteins participate in cancer, transcription, circadian clock, and Alzheimer diseases. O-GlcNAcylation has extensive cross-talk with phosphorylation and ubiquitination and takes part in numerous pathways. Many O-GlcNAcylation sites have been shown to occur adjacent to Ser/Thr sites known to be phosphorylated, thus competing for the same amino acids or posing a steric hindrance to the adjoining sites for other modifications.

The role of O-GlcNAcylation in the cell cycle has recently caught wide attention. Upon entering S phase, O-GlcNAcylation decreases globally. During mitosis, increased O-GlcNAc delays G2/M progression in mammalian cells. Inhibition of OGT prevents G2/M transition in Xenopus. Histone 3 is O-GlcNAcylated on Thr-32 during mitosis, which antagonizes phosphorylation at Ser-10. O-GlcNAc cycling regulates mitotic spindle organization, as increasing both OGT/OGA activity disrupts spindle structures. During cytokinesis, extensive cross-talk between O-GlcNAcylation and phosphorylation has also been observed. Specifically, the intermediate filament vimentin is O-GlcNAcylated at the midbody.

Here we identify O-GlcNAcylation occurring on Cdh1 (Cdc20 homologue 1; also known as Fzr1), a co-activator of anaphase promoting complex/cyclosome (APC/C). APC/C is a key multisubunit E3 ligase that orchestrates mitosis. Cdh1 engages with APC/C through an N-terminal C-box motif (DRFIP, amino acid 46–50) and a C-terminal Ile-Arg (IR) tail (12, 13). Cdh1 is subject to myriad modifications. It is phosphorylated at Ser-40, Thr-121, Ser-151, and Ser-163 by cyclin E-CDK or cyclin A-CDK complexes (14) and a total of nine sites (including the four sites above) by Cdk1 during M phase (15), which attenuates its interaction with other subunits of APC/C. It is also ubiquitinated by itself and mediates proteasome-dependent degradation during mitosis. Cdh1 engages with APC/C through an N-terminal C-box motif (DRFIP, amino acid 46–50) and a C-terminal Ile-Arg (IR) tail (12, 13). Cdh1 is subject to myriad modifications. It is phosphorylated at Ser-40, Thr-121, Ser-151, and Ser-163 by cyclin E-CDK or cyclin A-CDK complexes (14) and a total of nine sites (including the four sites above) by Cdk1 during M phase (15), which attenuates its interaction with other subunits of APC/C. It is also ubiquitinated by itself and mediates its own demise (16). Cdh1 maintains APC/C activity after anaphase and throughout G1 and prevents cell cycle re-entry until a cell commits to another round of cell division (11).

Besides the well documented role in mitosis, APC/C-Cdh1 is also pivotal for neuronal development (17). In the mammalian brain, both APC/C core subunits and Cdh1 are abundant in post-mitotic neurons (18). In the chick brain, multiple Cdh1 homologues have been identified (19). In both mammals and flies, only Cdh1, but not Cdc20, activates APC/C function in neurons (20, 21). This is in sharp contrast to mitotic regulation, where either Cdh1 or Cdc20 complexes with APC/C. A recent
embryo-restricted Cdh1 knock-out mouse model showcased that an APC/C-Cdh1 complex is required for neurogenesis and cortical neuron development (22). Because neurons are postmitotic, they need an active APC/C-Cdh1 complex to degrade mitotic cyclins to suppress aberrant cell cycle reentry and subsequent neuronal deaths by apoptosis (23). Meanwhile, O-GlcNAc modification is most abundant in the brain. We thus suspect a link between Cdh1 and O-GlcNAc. In this report we show that Cdh1 is O-GlcNAcylated. Using higher energy collision dissociation (HCD) mass spectrometry (MS), we identify the peptide that is potentially O-GlcNAcylated. We propose that O-GlcNAcylated Cdh1 antagonizes its phosphorylation and promotes APC/C-Cdh1 activity.

**Experimental Procedures**

**Cell Culture, Antibodies, and Plasmids**—HeLa cells were purchased from ATCC. They were grown in DMEM (Hyclone) containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C in a 5% CO2 incubator. Anti-OGT antibodies were from Santa Cruz (sc-32921), anti-O-GlcNAc antibodies (CDT110.6) were from Sigma, anti-O-GlcNAc antibodies (RL2) were from Abcam, anti-Cdc27 antibodies were from Bethyl Laboratories (BL4897), anti-phospho-Ser/Thr-Pro antibodies (MPM-2) were from Millipore (05-368), and anti-Cdh1 antibodies were from Abcam (ab3242). OGT was amplified by PCR to introduce an upstream BamHI site and a downstream ApaI site and then cloned into pcDNA3.0–3HA vectors, resulting in pcDNA-3HA-OGT-CDH1-4A (14), -4D (14), -540A, and -3A mutants were generated using specific primers (sequences available upon request) following the manufacturer’s instructions (QuickChange II, Stratagene). OGT-N (1–473) and -C (474–1046) plasmids were generated by PCR. siOGT oligos were GCAGGCUCUGAAACUAAdTdT, and the control siRNA oligos were CGUACCGGAAUACUUCGAdTdT (both from Dharmacco).

**Cell Culture Synchronization**—A double thymidine (an inhibitor of DNA synthesis) block was utilized for cell synchronization in S phase (Fig. 3A). For cells enriched in M phase, 340 nm/ml nocodazole (Noc) was added 9 h after release from the second thymidine block. 16 h later cells were collected.

**Immunoprecipitation (IP), Immunoblotting (IB), and Silver Staining**—IP and IB experiments were performed as described before (24). Where indicated, cells were treated with 340 nm/ml Noc for 14 h and then 2 μM Ro-3306 (CDK1 inhibitor) for 1 or 2 h (Fig. 3B). Noc for 16 h, 100 mM O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino N-phenylcarbamate (PUGNAc; OGA inhibitor) for 24 h, and 30 mM glucose for 3 h (Fig. 3D). The following primary antibodies were used for immunoblotting: anti-OGT, anti-O-GlcNAc, anti-Cdh1, anti-Cdc27, anti-β-actin, anti-HA, and anti-FLAG M2 (Sigma). For IP with anti-OGT, -Cdc27, and -HA antibodies, protein A-Sepharose was used; for IP with anti-Cdh1, -FLAG antibodies, protein G-Sepharose was used; for IP with anti-O-GlcNAc IgM CTD110.6 antibodies, anti-mouse IgM-agarose beads were used. Peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch. Blotted proteins were visualized using the ECL detection system (Amersham Biosciences). Signals were detected by an LAS-4000 and quantitatively analyzed by densitometry using the Multi Gauge software (Fujifilm). All Western blots were repeated for at least three times. The numbers reported for the densitometry were based on at least three independent experiments. Statistical significance was measured by Student’s t test.

For silver staining HeLa cells transfected with wide-type (WT) CDH1 were incubated with 100 μM PUGNAc for 24 h and 30 mM glucose for 3 h. Cell lysates were subject to IP using anti-FLAG agarose. The bound proteins were glycine-eluted. Separated protein bands in the SDS-PAGE gel were visualized with an MS-compatible silver stain kit (Proteosilver Plus, Sigma).

**HCD-MS Analysis**—Samples were precipitated with 4 volumes of ice-cold acetone, air-dried, resuspended in 8 m urea, 100 mM pH 8.5 Tris buffer, and digested with trypsin (Promega) at 1:50 enzyme/protein ratio. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed on an Easy-nLC 1000 UPLC (Thermo Fisher Scientific) coupled with a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific). Peptides were loaded on a precolumn (75-μm inner diameter, 8 cm long, packed with ODS-AQ 12 nm–10 μm beads from YMC Co., Ltd.) and separated on an analytical column (75-μm inner diameter, 11 cm long, packed with Luna C18 3 μm 100 Å resin from Phenomenex) using an acetonitrile gradient from 0 to 28% in 55 min at a flow rate of 200 nl/min. The top 10 most intense precursor ions from each full scan (resolution 70,000) were isolated for HCD MS2 (resolution 17,500; NCE 27) with a dynamic exclusion time of 20 s. Precursors with 1+, >5+, or unassigned charge states were excluded. A conventional database search using Prolucid (25) against a human database was performed with GlcNAc (203.079373) as a differential modification on Ser/Thr. Search results were filtered using DTASelect 2.0 with 10 ppm mass accuracy for precursor mass and a 1% false discovery rate cutoff at the spectral level (26). Spectra were labeled by pLabel with 20 ppm tolerance for fragment ions (27). GlcNAc specific reporters were also labeled (126.05495, 138.05495, 144.06551, 168.06552, 186.07608, 204.08665) (28).

**In Vitro APC/C Assay**—In vitro APC/C ubiquitination assays were carried out as described (29). FLAG-CDH1-WT or -3A plasmids were transfected into 293T cells. The cells were harvested 48 h later for lysis, immunoprecipitated with anti-FLAG agarose beads, washed, and eluted. About 15 μg of FLAG-Cdh1-WT or -3A proteins were incubated with anti-Cdc27 beads (5 μl) at room temperature for 1 h. Then the beads were washed followed by adding 5.5-μl reaction buffers with an energy mix (150 μM HA-ubiquitin (Boston Biochem), 5 μM GST-cyclin B1, 5 μM E1, 2 μM E2 UbCH5c). After 1 h of incubation at room temperature, the reaction was quenched with SDS sample buffers, loaded onto SDS-PAGE, and subjected to IB analysis.

**Results**

**Cdh1 Is O-GlcNAcylated**—As many nucleocytoplasmic proteins are modified dynamically by O-GlcNAc (30), we sought to assess whether Cdh1 is also regulated by such modifications. Because O-GlcNAcylation is extremely labile and easily escapes detection, the OGA inhibitor PUGNAc was utilized. HeLa cells...
 Flag-Cdh1 interacts with both OGT-TPR and -ED fragments, and co-IP experiments were carried out (Fig. 2). These data further implicate Cdh1 in the OGT/OGA co-IP between HA-OGT and FLAG-Cdh1 was also observed (Fig. 2). To exclude the possibility of the antibodies fortuitous co-IP, FLAG-Cdh1 was transfected into HeLa cells, and anti-FLAG immunoprecipitates were subject to the same analysis as in Fig. 1A. We observed O-GlcNAcylation occurring on FLAG-Cdh1 (Fig. 1C). As all these experiments were performed in cultured cells, we sought to identify whether the modification also occurs in vivo. As Cdh1 is the only activator of APC/C in the brain, we used mouse brain extracts and detected O-GlcNAcylated proteins identified to date (1) (Fig. 3A). We noted that Ser-39 conforms to the PVST (proline-valine-serine-threonine) motif that is conserved in about half of the O-GlcNAc group. Thus, we identified Ser-39/Ser-40/Ser-42 as the major O-GlcNAcylated sites: Ser-39, Ser-40, or Ser-42 (Fig. 3B). We used both RL2 and CTD110.6 antibodies, as they target to the potential O-GlcNAcylated site of Cdh1, we enriched O-GlcNAcylated by treating the cells with PUGNAc and glucose, then subject to IP and IB with the antibodies indicated. D, mouse brain extracts were subject to IP with anti-Cdh1 antibodies and to IB with the indicated antibodies.

**FIGURE 1. Cdh1 is O-GlcNAcylated.** A and B, HeLa cell extracts were subject to IP with anti-Cdh1 (A) or anti-O-GlcNAc (CTD110.6) (B) antibodies and blotted with the indicated antibodies. PUGNAc treatment was as indicated. Anti-mouse IgM-agarose beads were used in B. The bar graph represents densitometry analysis of IP experiments in A. The data are representative of four independent experiments. The asterisk indicates significant differences from control. C, HeLa cells were transfected with FLAG-Cdh1, treated with PUGNAc and glucose, then subject to IP and IB with the antibodies indicated. D, mouse brain extracts were treated with PUGNAc or mock-treated were subject to IP assays with anti-Cdh1 antibodies and blotted with anti-O-GlcNAc antibodies (CTD110.6). We detected a crisp O-GlcNAc band not only at positions corresponding to Cdh1 but also of increasing intensities upon PUGNAc treatment (Fig. 1A). Reciprocally, Cdh1 was also present in anti-O-GlcNAc antibody immunoprecipitates (Fig. 1B). To exclude the possibility of the antibodies’ fortuitous co-IP, FLAG-Cdh1 was transfected into HeLa cells, and anti-FLAG immunoprecipitates were subject to the same analysis as in Fig. 1A. We observed O-GlcNAcylation occurring on FLAG-Cdh1 (Fig. 1C). As all these experiments were performed in cultured cells, we sought to identify whether the modification also occurs in vivo. As Cdh1 is the only activator of APC/C in the brain, we used mouse brain extracts and detected O-GlcNAcylated in cell cultures and in vivo.

**Cdh1 Interacts with OGT**—The results above suggest that Cdh1 is subject to O-GlcNAcylation modification. Because mammals have only one OGT that catalyzes the reaction, we sought to examine whether Cdh1 associates with OGT. HeLa cell extracts were subject to IP with anti-OGT antibodies, and Cdh1 was identified in the immunoprecipitates (Fig. 2A). HA-OGT and FLAG-Cdh1 were transfected into HeLa cells, and IP assays were carried out with anti-FLAG antibodies. Again, the two proteins co-immunoprecipitated (Fig. 2B). Reciprocal co-IP between HA-OGT and FLAG-Cdh1 was also observed (Fig. 2C). These data further implicate Cdh1 in the OGT/OGA cycle.

As OGT has N-terminal 13.5 tetratricopeptide repeats (TPR) and a C-terminal enzymatic domain (ED) (31), we sought to determine which fragment of OGT interacts with Cdh1. OGT-TPR and -ED constructs were co-transfected with Cdh1 plasmids, and co-IP experiments were carried out (Fig. 2, D and E). FLAG-Cdh1 interacts with both OGT-TPR and -ED fragments, with the ED terminus displaying stronger interaction (Fig. 2E), suggesting that OGT indeed catalyzes Cdh1 O-GlcNAcylation.

**MS Identifies O-GlcNAcylation Sites of Cdh1**—To identify the potential O-GlcNAcylated site of Cdh1, we enriched O-GlcNAcylated by treating the cells with PUGNAc and glucose as described (32). Glucose treatment further enhanced O-GlcNAcylated levels of Cdh1 compared with PUGNAc treatment only (Fig. 1C). The same assay was carried out on a larger scale, and the samples were subject to silver staining (Fig. 3A) before HCD-MS analysis (Fig. 3B). Cdh1 bands were in-solution-digested with trypsin, and the peptides were analyzed by MS. With the sequence coverage of 85% of Cdh1, we identified one O-GlcNAc-modified peptide revealing three potential O-GlcNAcylated sites: Ser-39, Ser-40, or Ser-42 (Fig. 3B). We noted that Ser-39 conforms to the PVST (proline-valine-serine-threonine) motif that is conserved in about half of the O-GlcNAcylated proteins identified to date (1) (Fig. 3C).

To further validate the modification site, we constructed S39A, S40A, and S42A single mutants and a S39A/S40A/S42A (3A) triple mutant by mutagenesis and transfected them into HeLa cells. Although the WT Cdh1 elevated O-GlcNAcylated upon PUGNAc and glucose incubation, O-GlcNAcylation in the 3A mutant was significantly diminished (Fig. 3, D and E). We used both RL2 and CTD110.6 antibodies, as they target to distinct antigens, whereas both recognize the O-GlcNAc group. Thus, we identified Ser-39/Ser-40/Ser-42 as the major O-GlcNAcylated sites of Cdh1.

**O-GlcNAcylated of Cdh1 Antagonizes Its Phosphorylation**—Previous investigations have established that Cdh1 is phosphorylated by Cdk1 during mitosis at nine Ser/Thr sites, including the four sites shown in Fig. 4A, which dampens its interaction with other components of APC/C and inactivates APC/C/Cdh1 (14, 15). Moreover, the peptide we identified harbors Ser-40 (Fig. 3B), which is one of the phosphorylation sites (14, 15). Therefore, we examined O-GlcNAcylated of Cdh1 in the event...
of its phosphorylation. HeLa cells were blocked with thymidine before being released into Noc. Mitotic extracts were then analyzed by Western blotting. Echoing previous results, mitotic Cdh1 displays a distinct band migration compared with asynchronous cells (Fig. 4B), indicative of mitosis-specific phosphorylation. Intriguingly, the O-GlcNAcylation of Cdh1 decreased to ~40% during mitosis (Fig. 4B), suggesting that O-GlcNAcylation and phosphorylation of Cdh1 may be antagonistic of each other, reminiscent of the yin-yang model (1).

To further characterize the cross-talk between O-GlcNAcylation and phosphorylation, we aimed to prevent mitotic phosphorylation of Cdh1 by inhibiting Cdk1. We reasoned that O-GlcNAcylation of Cdh1 will be altered upon Cdk1 inhibition, even when cells are in the mitotic phase. HeLa cells were incubated with Ro-3306, a Cdk1 inhibitor (33), for 1 or 2 h after Noc-induced mitotic arrest. When only treated with Noc, the O-GlcNAcylation of Cdh1 was reduced (Fig. 4C). Upon Ro-3306 incubation, HeLa cells display higher levels of O-GlcNAcylation of Cdh1. Prolonged treatment of Ro-3306 further elevates O-GlcNAcylation of Cdh1 (Fig. 4C), suggesting that phosphorylation of Cdh1 counteracts O-GlcNAcylation.

To study the O-GlcNAcylation of phospho-mutants, we constructed the phospho-deficient 4A and phospho-mimic 4D mutants, as previous investigations established them as the major Cdk-dependent phosphorylation sites (14). HeLa cells were transfected with WT, 4A, and 4D plasmids, incubated with PUGNAC and glucose, then subject to Noc treatment (Fig. 4D). The phospho-deficient 4A mutant enhanced O-GlcNAcylation compared with WT, whereas the phospho-mimicking 4D mutant displayed lower levels of O-GlcNAcylation (Fig. 4E).

To directly visualize whether lacking OGT promotes Cdh1 phosphorylation, we used MPM-2 antibodies, which recognize a p(S/T)P phospho-epitope, characteristic of Cdk substrates. HeLa cells were treated with siOGT or mock-treated, transfected with FLAG-Cdh1, and subject to IP. Upon OGT inhibition, FLAG-Cdh1 immunoprecipitates manifest an elevated phosphorylation, discernable through MPM-2 IB (Fig. 4F). This is also in line with our hypothesis that O-GlcNAcylation offsets phosphorylation.

Given that OGT is essential for survival and, therefore, Fig. 4F might be an indirect effect, we directly tested the MPM-2 levels of Cdh1 mutants (Fig. 4G). HeLa cells transfected with Cdh1-WT, S40A, 3A, and 4A mutants were treated with Noc, then the MPM-2 levels were examined in the immunoprecipitates. As expected, the phospho-deficient S40A and 4A mutants significantly hampered Cdh1 phosphorylation (Fig. 4G). The 3A mutant enhanced MPM-2 levels compared with the S40A mutant (Fig. 4G), suggesting that O-GlcNAcylation indeed antagonizes phosphorylation.

O-GlcNAcylation of Cdh1 Enhances APC/C Activity—Given that phosphorylation of Cdh1 antagonizes the interaction between Cdh1 and APC/C subunits (14), we reasoned that O-GlcNAcylation may also be involved. HeLa cells were transfected with FLAG-Cdh1-WT, -S40A, -3A, or -4A plasmids, and then the interaction between an APC/C subunit, Cdc27, and Cdh1 was examined (Fig. 5A). Cdh1-S40A displayed stronger interaction with Cdc27, as Ser-40 is a known phosphorylation site. Interaction between Cdh1-4A and Cdc27 was even stronger, as the four phosphorylation sites were all mutated. The 3A mutant reduced interaction compared with S40A (Fig. 5A), suggesting that when O-GlcNAcylation was attenuated, Cdc27 binds with Cdh1 in a weaker fashion. The result indicates that O-GlcNAcylation promotes the interaction between Cdh1 and APC/C subunits.

Because OGT is the only enzyme that catalyzes O-GlcNAcylation in mammalian cells, we reasoned that the interaction between Cdc27 and Cdh1 will be compromised after OGT depletion. To that end, HeLa cells were treated with siOGT and transfected with FLAG-Cdh1. When Cdc27 immunoprecipitates

FIGURE 2. Cdh1 interacts with OGT. A, total HeLa cell extracts were subject to IP analysis with anti-OGT antibodies and blotted with the indicated antibodies. B and C, HeLa cells transfected with FLAG-Cdh1 and HA-OGT were subjected to IP with anti-FLAG (B) or anti-HA antibodies (C) and to IB with the antibodies indicated. D, a diagram of OGT protein structure and constructs used in this study showing N-terminal TPR repeats and the C-terminal ED. E, HeLa cells were transfected with HA-OGT-WT, TPR, or ED plasmids together with FLAG-Cdh1 then subjected to IP and IB analysis with the antibodies indicated.
were examined, FLAG-Cdh1 showcased a drastic reduction of Cdc27 interaction (Fig. 5B), consistent with our educated guess.

Then we sought to determine whether O-GlcNAcylation of Cdh1 underwrites APC/Cdh1 activity. We first tested whether OGT depletion affects cyclin B1 ubiquitination. HeLa cells were treated with si

![Image](image_url)

**FIGURE 3.** Mass spectrometry identifies one O-GlcNAcylated peptide of Cdh1. A, HeLa cells were transfected with FLAG-Cdh1, then enriched for O-GlcNAcylation as described (32); that is, treated with 100 mM PUGNAc for 24 h and 30 mM glucose for 3 h. Cell lysates were immunoprecipitated with anti-FLAG-agarose then subjected to silver staining (A). The FLAG-Cdh1 band was indicated. B, HCD mass spectrum identified an O-GlcNAcylated Cdh1 peptide. From this HCD spectrum, an O-GlcNAcylated peptide TLTPASSPVSSPSK of Cdh1 was identified, indicating that potential glycosylation occurs on Ser-39, Ser-40, or Ser-42 labeled by pLabel. b and y ion series represents fragment ions containing the N and C termini of the peptide, respectively. Ser-39, Ser-40, and Ser-42 are shown in red above the spectrum to indicate the potential glycosylation sites. C, Ser-39, Ser-40, and Ser-42 are evolutionarily conserved. D and E, HeLa cells were transfected with FLAG-Cdh1-WT, -S39A, -S40A, -S42A, and -S39A/S40A/S42A (3A) plasmids and then treated as in A. The anti-FLAG immunoprecipitates were subject to IB with CTD110.6 in (D) and with RL2 in (E). Bar graphs below D and E indicate O-GlcNAcylation levels normalized with WT. The data are representative of three independent experiments. Asterisks indicate significant differences from control.
proteins were first isolated from 293T cells and then incubated with GST-cyclin B1 in the presence or absence of anti-Cdc27 beads (Fig. 5D). The in vitro APC/C ubiquitination results suggested that cyclin B1 ubiquitination decreased in the 3A mutant, indicative of compromised APC/C activity. Hence, O-GlcNAcylation of Cdh1 underscores APC/C\(_{\text{Cdh1}}\) activity.

**Discussion**

Cdh1 is a critical activator of APC/C, and no wonder its regulation comes in many different flavors: acetylation (34), ubiquitination (16, 35, 36), and phosphorylation (14, 15). Now added into the list of modifications is O-GlcNAcylation. Our study reveals that Cdh1 interacts with OGT, and Cdh1 is O-GlcNAcylated (Figs. 1 and 2). Consistent with our results, Cdh1 was recently identified in a screen using in vitro OGT assays on substrate peptides derived from annotated protein kinase substrates (37).

By HCD-MS analysis, we mapped the modification sites to Ser-39, Ser-40, and S42, although we cannot conclude all three sites are O-GlcNAcylated in vivo. It is difficult to determine which single amino acid is O-GlcNAcylated, but the 3A mutant compromised O-GlcNAcylation levels using both RL2 and CTD110.6 antibodies (Fig. 3, D and E). As OGT is known to be
promiscuous in catalyzing adjacent sites (38, 39), it is possible that mutating one of the Ser-39, Ser-40, or Ser-42 sites will result in O-GlcNAcylation on adjoining sites. We also noted that Ser-39, Ser-40, and Ser-42 contains a PVS motif that has been identified in half of the O-GlcNAcylated sites (Fig. 3C) (1), and the three single mutations induced increased O-GlcNAcylation with the CTD110.6 antibodies (Fig. 3D). This seems paradoxical at the first glimpse, but it also has been observed in other O-GlcNAcylated proteins (39). It is conceivable that Ser-39, Ser-40, or Ser-42 are the most preferred O-GlcNAcylation sites without which OGT lingers longer with Cdh1, resulting in increased O-GlcNAcylation. In addition, our results suggest that there might be other O-GlcNAcylation sites, as the 3A mutant did not completely abolish the O-GlcNAcylation signals (Fig. 3D and E).

We cannot help but notice that Ser-40 is one of the sites previously identified to be phosphorylated by CDK (14). It could be that Ser-40 is reciprocally occupied by the O-GlcNAc or phosphate group at distinct cell cycle stages. This is the case for c-Myc, RNA polymerase II, and other proteins (1). Alternatively, O-GlcNAc could compete for Ser-39/Ser-42 to poise a steric hindrance to phosphorylation at Ser-40. This proximal competition scenario has also been observed for vimentin, p53 and CAMKIV (1). We cannot preclude one model from the

FIGURE 5. O-GlcNAcylation of Cdh1 activates APC/C activity. A, HeLa cells were transfected with FLAG-Cdh1-WT, -S40A, -3A, or -4A, then subject to IP with IgG or Cdc27 antibodies. B, HeLa cells were treated with siOGT or mock-treated, transfected with FLAG-Cdh1, and then subjected to IP with antibodies indicated. C, HeLa cells were treated siOGT or mock-treated, transfected with HA-Ub (HA-ubiquitin), and then subjected to IP with the antibodies indicated. D, in vitro APC/C assay using cyclin B1 ubiquitination as a reporter in the presence of WT or O-GlcNAc-deficient 3A forms of Cdh1 purified from 293T cells. Lane 1, reaction mix with Cdh1-WT but without anti-Cdc27 beads. Lane 2, reaction mix with anti-Cdc27 beads but without Cdh1. Lane 3, reaction mix with both Cdh1-WT and anti-Cdc27 beads. Lane 4, reaction mix with O-GlcNAc-deficient Cdh1–3A and anti-Cdc27 beads. Bar graphs below A–D indicated densitometric quantitation of Western blot bands. The data are representative of three independent experiments. Asterisks indicate significant differences from control. Dots represent significant differences between the indicated samples. E, a proposed model of Cdh1 O-GlcNAcylation. O-GlcNAcylation antagonizes phosphorylation of Cdh1, where the former promotes the interaction between Cdh1 and APC/C and the latter inhibits the Cdh1-APC/C interaction.
other at the moment; further electron transfer dissociation mass spectrometry analysis will be needed to pinpoint the exact O-GlcNAcylated residues. Our results suggest that O-GlcNAcylation antagonized phosphorylation of Cdh1 and promoted interaction between Cdh1 and APC/C subunits and thus APC/C-Cdh1 activity (Fig. 5E).

Now we come to a very interesting scenario; acetylation, phosphorylation, and ubiquitination of Cdh1 all aim to negatively regulate APC/C-Cdh1 activity, although the first two do so by preventing Cdh1-APC/C binding and the last one does so by down-regulating Cdh1 protein levels. O-GlcNAcylation of Cdh1 is the only modification hitherto identified to promote Cdh1-APC/C interaction. How does O-GlcNAc achieve that? A most recent analysis of atomic structures of the APC/C complex suggests that phosphorylation of Cdh1 at Ser-40, Thr-121, Ser-151, and Ser-163 may poise electrostatic repulsion (13), and we speculate that O-GlcNAc modification may partially alleviate the repulsion by blocking phosphorylation at Ser-40. On the other hand, Ser-39/Ser-40/Ser-42 is immediately adjacent to the N-terminal C-box (DRFIP, amino acid 46–50) that is essential for interaction between Cdh1 and APC/C (12). O-GlcNAc at proximal sites may promote the interaction between the C-box and APC/C.

Of the two activators of APC/C, only Cdh1 is present in neuronal cells. APC/C-Cdh1 activity has been linked to neuronal survival, APC/C-Cdh1 activity is elevated in postmitotic neurons (18), Cdh1 silencing triggers neuronal cell deaths via apoptosis (40, 41), and Cdh1 is required to prevent cyclin B1 accumulation in neurons (40, 41). Intriguingly, most abundant in the brain is O-GlcNAc modification, and mounting evidence suggests that O-GlcNAcylation is involved in Alzheimer disease pathology (1, 42). We envision that reduced O-GlcNAc renders lower activity of APC/C-Cdh1, hence higher cyclin B1 levels, entailing the post-mitotic neurons to cell cycle reentry.

Author Contributions—J. Li wrote the manuscript. J. Li, M.-Q. D., and X. X. designed the project and analyzed the data. J. T., Q. G., and J. Liao performed the biochemical assays. Y. D. carried out the experiments, and X. X. designed the project and analyzed the data. J. T., Q. G., and X. X. also coordinated the project.

Acknowledgments—We thank Dr. Tieshan Tang for mice, Dr. Hongtao Yu for sharing reagents and protocols, and members of the Xu laboratory for helpful discussion and comments on the manuscript.

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