Perturbations in O-linked β-N-Acetylglucosamine Protein Modification Cause Severe Defects in Mitotic Progression and Cytokinesis* §

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The dynamic modification of nuclear and cytoplasmic proteins with O-linked β-N-acetylglucosamine (O-GlcNac) is a regulatory post-translational modification that is rapidly responsive to morphogens, hormones, nutrients, and cellular stress. Here we show that O-GlcNac is an important regulator of the cell cycle. Increased O-GlcNac (pharmacologically or genetically) results in growth defects linked to delays in G2/M progression, altered mitotic phosphorylation, and cyclin expression. Overexpression of O-GlcNacase, the enzyme that removes O-GlcNac, induces a mitotic exit phenotype accompanied by a delay in mitotic phosphorylation, altered cyclin expression, and pronounced disruption in nuclear organization. Overexpression of the O-GlcNac transferase, the enzyme that adds O-GlcNac, results in a polyploid phenotype with faulty cytokinesis. Notably, O-GlcNac transferase is concentrated at the mitotic spindle and midbody at M phase. These data suggest that dynamic O-GlcNac processing is a pivotal regulatory component of the cell cycle, controlling cell cycle progression by regulating mitotic phosphorylation, cyclin expression, and cell division.

Because of the discovery of cyclins 22 years ago (1), a working model of the cell cycle has slowly been constructed. The cell cycle oscillator is well orchestrated cell division (2). Nevertheless, a detailed mechanism of the cell cycle has slowly been constructed. The cell cycle oscillator is

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§ The abbreviations used are: O-GlcNac, O-linked β-N-acetylglucosamine; OGT, O-GlcNac transferase; PUGNAc, phenylurethane of O-GlcNac; DON, 6-diazo-5-oxo-L-norleucine; pRB, retinoblastoma protein; YY1, Yin Yang 1; GFP, green fluorescent protein; PBS, phosphate-buffered saline; OGlcnAcase, β-N-acetylglucosaminidase.
O-GlcNAc Is a Dynamic Regulator of the Cell Cycle

**RESULTS**

Dynamic O-GlcNAc Protein Modification Is Required for Cell Growth—To test the hypothesis that dynamic O-GlcNAc protein modification is a control mechanism for cellular growth, we raised global levels of O-GlcNAc using the O-GlcNAcase inhibitor PUGNAc and determined the growth rate of multiple cell lines. Cell number was measured daily; differences between control and PUGNAc-treated cells were evident at 4 days and pronounced at 5 days (Fig. 1A). In almost every cell line studied, elevated O-GlcNAc levels reduced the cells growth rate (Fig. 1A). However, logarithmically growing 3T3-L1 cells showed only a slight inhibition of growth upon chronic PUGNAc treatment. This is most likely due to the low permeability of PUGNAc in undifferentiated 3T3-L1 cells.

O-GlcNAc- and Proline-directed Phosphorylation Are Reciprocal at M Phase—Next, we determined if O-GlcNAc levels were regulated in a cell cycle-dependent manner. HeLa cells were mitotically arrested at the metaphase-anaphase transition with nocodazole (400 ng/ml, 12 h) (33). O-GlcNAc-modified proteins in total cell extract were determined by immunoblot with an O-GlcNAc-specific antibody (29). O-GlcNAc staining was decreased compared with asynchronous control (Fig. 1B,
O-GlcNAc Is a Dynamic Regulator of the Cell Cycle

FIGURE 1. Growth delays in cells with elevated O-GlcNAc. A, chronic treatment of mammalian cell lines with PUGNAc (100 μM every 12 h) causes delays in cell growth as judged by alamarBlue proliferative dye assay. Control cells are black, and PUGNAc-treated cells are blue. B, nocodazole synchronization of HeLa cells shows increased staining in total cell extracts for mitotic phosphorylation (left panel) and decreased O-GlcNAc staining (right panel) by Western blot (WB) analysis. Lanes marked C are asynchronous control cells, and lanes marked N are nocodazole-synchronized. Actin is shown as a loading control. Proteins whose 110.6 staining decreases upon synchronization are marked with blue arrows, whereas proteins with increased staining are marked with red arrows. C, YY1 was immunoprecipitated (Ip) from asynchronous control (C) or nocodazole (N)-treated HeLa cells. The protein expression of YY1 is constant, but the levels of O-GlcNAc increase on the high molecular weight band of YY1 after nocodazole treatment. A rabbit nonspecific antibody control (1°) and a YY1 antibody control (1°) are included. D, nocodazole-synchronized HeLa cells treated with PUGNAc demonstrate decreases in OGT expression and increases in O-GlcNAcase expression. Lanes marked C are control cells, and lanes marked P are PUGNAc-treated cells. Three replicate samples are shown with actin-loading controls and 110.6 controls to validate the increase in O-GlcNAc levels with PUGNAc.

right panel). Although the overall O-GlcNAc levels decrease in these cells and O-GlcNAc staining on a few proteins completely disappears (see blue arrows), an increase in O-GlcNAc staining is seen on several proteins (see red arrows).

We next determined the level of proline-directed phosphorylation using the antibody MPM-2, whose staining is known to increases during M phase (34). As expected the M-phase-arrested cells (nocodazole-treated) show a dramatic increase in the levels of MPM-2 reactivity (Fig. 1B, left panel). Sensitive Coomassie Blue staining of gels indicates that the polypeptide levels of most of the O-GlcNAc-modified or M-phase-phosphorylated proteins does not change (data not shown). To determine whether O-GlcNAc and phosphorylation were reciprocal, we measured MPM-2 reactivity in cells treated with PUGNAc. Because PUGNAc inhibits O-GlcNAcase, the enzyme responsible for removing the sugar modification, we expected the increase in overall O-GlcNAc levels in both asynchronous and nocodazole-treated cells. Although PUGNAc increased the extent of 110.6 staining in many proteins in nocodazole-treated cells, PUGNAc treatment did not alter the distribution of O-GlcNAc-modified proteins in these cells. Proline-directed phosphorylation is not perturbed. Although no effect of PUGNAc was seen on proline-directed phosphorylation, we cannot rule out changes on specific proteins not resolved at the one-dimensional level.

To confirm that O-GlcNAc levels change on individual low abundance proteins and not the levels of the polypeptides themselves, we looked at two regulatory proteins already known to be O-GlcNAc-modified, Sp1 (35) and YY1 (Fig. 1C) (36, 37). When YY1 is immunoprecipitated from asynchronous and nocodazole-treated HeLa cells, the levels of O-GlcNAc on this transcription factor change (Fig. 1C). Nocodazole treatment causes an increase in 110.6 staining in a higher molecular weight band of YY1 and a decrease in 110.6 staining in a lower molecular weight band. When YY1 is immunoprecipitated from PUGNAc/nocodazole-synchronized cells, the extent of O-GlcNAc modification significantly increases, but the electrophoretic pattern is similar to untreated cells. The protein expression of YY1 is constant, and the 110.6 staining is completely abolished when 100 mM N-acetylglucosamine is added to the primary antibody mixture (data not shown). The Sp1 also shows an increase in glycosylation at M phase with no change in the protein expression (data not shown).

Interestingly, when HeLa cells are treated with PUGNAc, the levels of the O-GlcNAc-processing enzymes change (Fig. 1D). Triplicate samples are shown after 18 h of PUGNAc treatment in which OGT levels decline and O-GlcNAcase samples increase.

Pharmacological Modulation of O-GlcNAc Levels Disorganizes Cell Cycle Progression—To delineate the role of O-GlcNAc in cell cycle regulation, we measured the rate of cell cycle progression by flow cytometry in synchronized HeLa cells (double thymidine block). At the second thymidine block cells were treated with PUGNAc (100 μM) to raise O-GlcNAc levels or with 6-diazo-5-oxonorleucine, DON, (10 μM) to
lower levels. DON reduces O-GlcNAc levels due to its ability to inhibit glutamine fructose-6-amidotransferase, the rate-controlling enzyme of the hexosamine biosynthetic pathway, which produces the substrate for OGT (38, 11).

Cells with elevated O-GlcNAc (PUGNAc treatment) consistently demonstrate a delay in G2/M progression (Fig. 2A). In control cells almost 50% of the population had progressed through M phase and returned to G1 phase 10 h after release. In contrast, only 20% of PUGNAc-treated cells had returned to G1. Conversely, cells with decreased O-GlcNAc (DON) progress through the cell cycle at an accelerated rate compared with control (Fig. 2B). DON-treated cells moved through S phase faster, and by 14 h post-release 81% of the cells had returned to G1.

**FIGURE 2. Disruption of O-GlcNAc levels leads to cell cycle defects by flow cytometry.** A. HeLa cells were double thymidine blocked at the G1/S checkpoint and released. Before release, cells were incubated in PUGNAc (100 μM) for 12 h and at time of release. By flow cytometry analysis, PUGNAc-treated cells exhibit delayed M phase progression (left column, control; right column, PUGNAc). B. HeLa cells were treated as above with 10 μM DON. These cells rapidly progressed through S phase (left column, control; right column, DON). C. DON accelerates DNA synthesis as determined by [3H]thymidine incorporation (red, control; blue, DON; green, PUGNAc). All experiments were performed a minimum of three times and were highly reproducible.
phase compared with only 53% in controls. Glucosamine is a down-
stream metabolite of glutamine fructose-6-amidotransferase and
should ablate the effect of DON. When 10 mM glucosamine is added
with DON, cells progress at rates similar to control or PUGNAc-treated
cells (Supplemental Fig. 1).

To determine whether DON treatment accelerated S-phase progres-
sion, DNA synthesis was measured in synchronized HeLa cells by
\[^{3}H\]thymidine incorporation (Fig. 2C). DON-treated cells reached
maximal thymidine incorporation at 4 h, whereas control peaked at 5 h,
and PUGNAc-treated cells peaked at 6 h.

Next, we repeated these experiments in an independent model of cell
cycle synchronization that does not rely on chemical block. 3T3-L1
preadipocytes cells when grown to confluency become growth-quies-
cent. These cells are hormonally induced to differentiate into mature
adipocytes. Induction of differentiation is first characterized by two
rounds of synchronized cell cycle progression called mitotic clonal
expansion (39). Quiescent preadipocytes cells were treated with 100 \(\mu M\)
PUGNAc or 25 \(\mu M\) DON 12 h before induction of mitotic clonal expan-
sion and again at induction. Cells with elevated O-GlcNAc (PUGNAc-
treated) consistently showed significant delays in cell cycle progression
compared with non-treated controls (Fig. 3A). The delay corresponded
with the results seen earlier in the HeLa cells (Fig. 2).

Cells treated with DON failed to escape the \(G_0/G_1\) phase of the cell
cycle. The addition of DON to 3T3-L1 cells at the time of mitotic clonal
expansion induction or 12 h post-release in late \(G_1\) failed to escape \(G_1\).
However, DON added at 16 h (start of S phase) post-release did not

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**FIGURE 3.** 3T3-L1 cells show delayed cell cycle progression upon elevated O-GlcNAc. A, 3T3-L1 cells were released into mitotic clonal expansion by the addition of 0.5 mM
3-isobutyl-1-methylxanthine, 0.25 \(\mu M\) dexamethasone, and 10 \(\mu g/ml\) insulin. Cells treated with 25 \(\mu M\) DON 12 h before and at release fail to escape \(G_1\) phase, whereas 100 \(\mu M\)
PUGNAc-treated cells showed significant delays in S and \(G_2/M\) phase progression as judged by flow cytometry. B, elevated O-GlcNAc levels (PUGNAc treatment) caused a reduction of OGT expression, whereas O-GlcNAcase expression was higher. Conversely, lower O-GlcNAc levels (DON treatment) caused higher OGT expression and lower O-GlcNAcase expression. A 110.6 blot is shown showing the effects of DON and PUGNAc on O-GlcNAc levels, and actin is shown as a loading control. Each experiment was performed a minimum of three times. WB, Western blot.
interfere with 3T3-L1 cell growth (data not shown). This is consistent with HeLa cells, which show defects in G1 to S phase transition when treated with DON (data not shown).

Similar to HeLa cells, PUGNAc treatment altered the expression of O-GlcNAcase and OGT (Fig. 3B). Elevated O-GlcNAc by PUGNAc treatment caused a decrease in the expression of OGT and an increase in O-GlcNAcase expression. The inverse was seen after DON treatment.

**O-GT and O-GlcNAcase Control G2/M cell Cycle Progression**—Next, we decided to modulate the levels of the O-GlcNAc-processing enzymes, O-GlcNAcase (removes O-GlcNAc), and O-GlcNAc transferase (adds O-GlcNAc) using adenoviral infection. Large increases in the expression of vO-GlcNAcase and vOGT are toxic to most cells. Therefore, a multiplicity of infection that only caused a slight increase or decrease in O-GlcNAc levels was used.

Uninfected (data not shown) and GFP-infected cells exit G1/S and reach G2/M phase between 9 and 12 h, and by 15 h a large majority of the cells had returned to G1 phase (Fig. 4A). In the vO-GlcNAcase-infected cells, a significant defect in M phase progression was observed (Fig. 4A). At 15 h, when more than 65% of control cells had returned to G1 phase, 55% of vO-GlcNAcase-infected cells were still in M phase. This result was repeatedly seen in at least five independent experiments. Interestingly, a slight decrease in the length of S phase was observed in vO-GlcNAcase-infected cells, but the difference to GFP cells was not significant. Similar to vO-GlcNAcase, vOGT-overexpressing cells exhibit a prolonged M phase, but these cells also display severe aneuploidy (Fig. 4B). Upon release from the G1/S block, ~20% of the cells showed signs of aneuploidy, and the aneuploid nature of the cells increased upwards of 30% as the cells progressed through S and M phase. Uninfected and GFP-expressing cells showed only a small population of aneuploid cells (~2%).

**Overexpression of O-GlcNAcase and O-GlcNAc Transferase Disrupt Mitotic Phosphorylation**—Next, the effect of overexpressing OGT and O-GlcNAcase on O-glycosylation and mitotic phosphorylation was assessed. Overall levels of O-GlcNAc decrease in vO-GlcNAcase-infected cells ~10–15% (Fig. 5A, left panel), whereas overexpressed vOGT causes a slight increase in O-GlcNAc levels (Fig. 5B, left panel) and a substantial increase on an unidentified 55 kDa protein appears. Proline-directed phosphorylation, as determined by MPM2 staining, is reduced, and maximal phosphorylation lags compared with GFP cells when vO-GlcNAcase is overexpressed (Fig. 5A, right panel). The red box in the right panel of Fig. 5A is a longer exposure showing the dramatic difference in proline-directed phosphorylation after vO-GlcNAcase treatment in the 45–75-kDa range. When vOGT is overexpressed, cells never reach maximal mitotic phosphorylation; however, a 70-kDa band appears phosphorylated throughout the time course and is not present in the uninfected and GFP lanes (Fig. 5B, right panel). At G1/S, vO-GlcNAcase- and vOGT-infected cells are in a higher percentage of

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2 C. Slawson, N. E. Zachara, K. Vosseller, W. Cheung, M. D. Lane, and G. W. Hart, unpublished information.
M phase than GFP (Fig. 4, A and B, at time 0) as judged by flow cytometry; hence, the level of proline-directed phosphorylation is higher (Fig. 5, A, red panel, and B). Both mitotic kinases cyclin-dependent kinase 1 and 2 were assayed for activity (Supplemental Fig. 2) from vO-GlcNAcase-infected cells, and no dramatic changes in the level of activity during the cell cycle is found, suggesting overexpression is not affecting in vitro cyclin-dependent kinase activity.

The expression of the key G1/S phase regulator protein pRb is unaffected by viral infection (Fig. 6). This was not surprising since the levels of this protein remain constant during the cell cycle. The pRb protein is regulated by phosphorylation on threonine 826 (ppRb-T826) at late G1 by a cyclin-dependent kinase 4-cyclin D complex and disrupts its binding to E2F family proteins (40, 41). The vO-GlcNAcase-infected cells show delayed and decreased pRb phosphorylation when compared with uninfected/GFP-infected cells (Fig. 6). Interestingly, vOGT-infected cells reach normal levels of phosphorylation at ppRB-T826, but staining fails to decline during the time course.

**Cyclin Expression Is Altered after Modulation of O-GlcNAc Levels**—Next, expression levels of several cyclins were measured. In GFP-infected cells, cyclins A and B show peak expression during M phase (9–12 h post-release) and a subsequent decline post-M phase (Fig. 7A). Interestingly, expression of cyclins A and B was prolonged in 3T3-L1 cells treated with PUGNAc (Fig. 7B). A similar pattern of cyclin expression was seen in HeLa cells after PUGNAc treatment (Supplemental Fig. 3).

In GFP and uninfected cells, cyclin E expression is maximal at release, declines throughout M phase, and increases again as cells enter G1. In the vO-GlcNAcase-infected cells, cyclin E protein levels decline at a slightly faster rate than control/GFP cells upon thymidine release; however, as the cells reentered G1, little cyclin E expression is seen. Cyclin E expression is severely depressed in vOGT-overexpressing cells (Fig. 7A). The G1 cyclin D in both sets of infected cells never fully reaches maximal expression compared with GFP controls. Normally, the expression of the cyclin-dependent kinase inhibitor p21 increases in cells as they enter G1, but p21 fails to express in both vO-GlcNAcase- and OGT-overexpressing cells (Fig. 7A). Cyclins are regulated by cellular localization (2), but the viral treatments appear to have little effect on cytolocalization of the cyclins (Supplemental Fig. 4).

**Increased O-GlcNAc Transferase Levels Causes Abnormal Cytokinesis and Aneuploidy**—We further explored the M phase defects using confocal microscopy to determine the cytolocalization of O-GlcNAcase and OGT at G1/S and M phase and what effects overexpression of these enzymes have on α-tubulin and DNA morphology. Synchronized HeLa

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**FIGURE 5.** vOGT/vO-GlcNAcase control mitotic phosphorylation. A, Western blots (WB) of vO-GlcNAcase-infected cells have reduced O-GlcNAc levels and delayed mitotic phosphorylation (left box, O-GlcNAc staining; right box, MPM2 staining). The red box is a longer MPM2 exposure of the 40–75-kDa range of the vO-GlcNAcase-infected cells. B, Western blots of vOGT-infected cells have increased O-GlcNAc and a sustained level of mitotic phosphorylation (left box, O-GlcNAc staining; right box, MPM2 staining).
FIGURE 6. Phosphorylation of pRb is disrupted by vOGT/vO-GlcNAcase. pRb phosphorylation at threonine 826 fails to reach maximum levels with vO-GlcNAcase infection and stays phosphorylated longer in vOGT-infected cells as judged by Western blotting (WB). The overall protein levels of pRb are unchanged by viral infection (bottom panel).

FIGURE 7. vOGT/vO-GlcNAcase expression extend cell cycle-regulated protein expression. A and B, levels of cyclins in synchronized cells were determined by Western blot (WB) analysis on equal amounts of total cell extract. A, panels showing expression of O-GlcNAcase, OGT, GFP, and actin. Cell cycle-regulated proteins cyclin A, B, E, D, and p21 expression is disrupted with vO-GlcNAcase or vOGT infection. B, Western blots of 3T3-L1 cells show failed cyclin A and B expression after 25 μM DON-treatment, and 100 μM PUGNAc treatment causes prolonged expression of cyclins A and B. The pattern of cyclin staining in each sample is highly reproducible.
cells were stained for DNA (red), α-tubulin (green), and either O-GlcNAcase or OGT (blue). At G1/S, control cells predominantly exhibited cytoplasmic staining for O-GlcNAcase and nuclear/perinuclear staining for OGT (Fig. 8A). At M phase, O-GlcNAcase appears ubiquitous throughout the cell, but the enzyme is mostly excluded from the nascent nuclear membranes of the daughter cells (Fig. 8B). Two different antibodies against O-GlcNAcase and OGT were used, and each antibody set gave the same result (Supplemental Fig. 5). Additionally, the preimmune sera for each antibody had little to no staining (Supplemental Fig. 5).

When vO-GlcNAcase is expressed, the cell morphology dramatically changes. First, the DNA appears more condensed compared with controls, and the α-tubulin network is dramatically perturbed. The cells tend to round up, and the α-tubulin forms a meshwork around the nucleus (Fig. 8A, second column, see arrow). At least 20% of the infected cells consistently show this phenotype. Previous work suggested that α-tubulin is modified by O-GlcNAc (42); therefore, vO-GlcNAcase overexpression may alter O-GlcNAc levels on tubulin and disrupt proper function.

In uninfected cells, OGT staining appears ubiquitous throughout the cell during interphase, but OGT localization is concentrated with the mitotic spindle during M phase (Fig. 9). At prophase, OGT becomes concentrated during the nascent formation of the mitotic spindle. A clearly defined increase in localization with the spindle is seen during metaphase. As the daughter chromosomes appear to pull apart during anaphase, OGT is found concentrated at the central spindle assembly. As the cleavage furrow forms during cytokinesis, OGT becomes concentrated at the midbody.

HeLa cells expressing vOGT exhibited multiple phenotypes at G1/S. Approximately 15–20% of the cells are aneuploid, whereas other cells still have visible midbodies (Fig. 8A, far right column, see arrow). A small percentage of cells round up like the vO-GlcNAcase-infected cells, but no visible condensation of the DNA is present nor does the α-tubulin network appear concentrated in a concentric ring around the nuclear membrane.

Co-localization of OGT with α-tubulin was observed by the presence of a light green color around the spindle and later within the midbody (Fig. 8B, column 1, see the arrow). When vOGT is overexpressed, a large population of cells (∼20%) repeatedly shows abnormal midbodies. Increased midbody staining is seen with even more OGT concentrated in this region; the α-tubulin polymers at the midbody appeared larger, and midbodies fail to separate and break off. The actin ring at the midbody cleavage furrow is intact, but the cells still fail to cytokinase properly (Supplemental Fig. 6).

**DISCUSSION**

Cell growth and division are the result of carefully coordinated events, which are regulated by protein expression, protein degradation, and protein phosphorylation. In this study we show that dynamic intracellular glycosylation, O-GlcNAc, also plays a role in cell cycle progression and cell division. The salient findings of this paper include the following: 1) Elevating or reducing O-GlcNAc levels results in changes in cell cycle length; 2) pharmacological modulation of global O-GlcNAc levels induces changes in the expression of OGT and O-GlcNAcase; 3) overexpression of vOGT or vO-GlcNAcase disrupts mitotic phosphorylation and the proper, timed expression of cyclin proteins; 4) OGT localizes to the mitotic spindle and midbody during cell division; 5) overexpression of OGT results in aneuploidy, due to defective cytokinesis.

**Dynamic Glycosylation and Phosphorylation Regulate Cell Cycle Progression**—Pharmacological increase (PUGNac) in O-GlcNAc causes cell growth delays in multiple cell lines. Previously, PUGNac was reported to have no effect on growth rates (27). In this study cells were treated with 100 μM PUGNac every 12 h, whereas it was administered every 48 h in the previous study. The effect on growth may result in changes to cell cycle length. Flow cytometry analysis on PUGNac-treated HeLa and 3T3-L1 cells support this observation (Figs. 2 and 3). These data concurred with previous reports demonstrating that increased UDP-GlcNAc levels slowed cell growth and differentiation in human colon cancer cells (43). A good example of this model was seen in *Xenopus laevis* oocytes (44). Several nuclear pore proteins are modified by O-GlcNAc (45), and these proteins are phosphorylated as oocytes progress from S to M phase (44). These proteins are mutually modified by O-GlcNAc and O-phosphate (44), but when O-GlcNAc proteins are capped with galactose by microinjection galactosyltransferase, severe defects in M- to S-phase transition occurs (25). Furthermore, when oocytes are preincubated in PUGNac before hormone-induced maturation, nuclear envelope breakdown is significantly delayed (28).

Like proline-directed phosphorylation, the O-GlcNAc protein modification is regulated in a cell cycle-dependent manner. Global O-GlcNAc levels after nocodazole synchronization decreased during M phase, when proline-directed phosphorylation, a hallmark of mitosis, peaks. However, an increase in glycosylation is seen on some protein bands. Previous work did not see changes in glycosylation after nocodazole synchronization (33); however, our study employed the 110.6 antibody that is highly specific for the O-GlcNAc modification.

In support of this, we looked at the glycosylation and expression status of Sp1 and YY1. YY1 is ubiquitously expressed transcription factor that is heavily modified by post-translational modifications including phosphorylation, acetylation, and O-GlcNAc (36, 37, and 46). O-GlcNAc levels on YY1 appear dynamically responsive in a cell cycle-dependent manner, possibly influencing specific protein-protein interactions and gene transcription. When YY1 is O-GlcNAc-modified, it is unable to associate with pRb-promoting increased binding of YY1 to specific promoters (36). Maximal binding of YY1 to pRb is found at G0/G1 (36). In this study we observed an increase in YY1 O-GlcNAc modification at M phase (nocodazole synchronization), which is when pRb is hyperphosphorylated and unable to interact with promoters. Additional support for dynamic cycling of the sugar modification can be found in COP11 protein cycling. COP11 proteins (specifically Sec24p) involved in endoplasmic reticulum-Golgi transport are phosphorylated at M phase, disrupting membrane binding; conversely, during interphase the protein is modified by O-GlcNAc and is functional (47). Of course, not all O-GlcNAc modified proteins demonstrate a decrease in O-GlcNAc at M phase. Previously, glycosylation of keratin 18 increased after nocodazole synchronization, whereas keratin 8 demonstrated an increase in phosphorylation after the same treatment (48, 33).

Lowering O-GlcNAc levels with DON leads to defects in G1/G0 progression. Although 3T3-L1 cells show G0/G1 arrest, HeLa cells show defects in G1 progression after DON treatment. These data are supported in OGT knockouts of fibroblast. Cells show delays in cell cycle progression and eventually become quiescent (22). Together, these data support a model in which low nutrients would result in low O-GlcNAc levels, and this would induce G0/G1 cell cycle arrest until nutrients are available again. Cells are most sensitive to low nutrient levels before G1 release since most of G1 is spent preparing the cells for replication and division (2).

Consistent with dynamic modulation of O-GlcNAc levels regulating cell cycle progression, once past the G1 checkpoint, lowering O-GlcNAc
FIGURE 8. vOGT/vO-GlcNAcase-expressing cells demonstrate mitotic exit phenotypes. A and B, confocal microscopy on triple-labeled synchronized HeLa cells. DNA is red, α-tubulin is green, and OGT/O-GlcNAcase is blue. A, cells at G1/S show cytoplasmic labeling for O-GlcNAcase and perinuclear labeling for OGT. O-GlcNAcase adenoviral infection causes condensed packaging of chromatin and a rounded phenotype. OGT-expressing cells show polyploid nuclei and improper cytokinesis. A white arrow points at the presence of a midbody in the vOGT-infected cells, and another white arrow points at the rounded α-tubulin network in the vO-GlcNAcase-infected cells. B, M-phase cells show localization of OGT to the mitotic spindle and midbody. O-GlcNAcase-expressing cells divide normally albeit slower, whereas OGT-expressing cells have thickened midbodies and disrupted cytokinesis. A white arrow points at OGT localization at the midbody in the control cells.
levels with DON accelerates S phase. A potential explanation for accelerated DNA synthesis is that O-GlcNAc-modified proteins are concentrated at condensed chromatin compared with transcriptionally active regions (49). As DNA is replicated, transcriptionally active euchromatin is replicated before the more condensed heterochromatin (50). O-GlcNAc on chromatin proteins might promote condensation of DNA. Histone deacetylase 1, which deacetylates histones and promotes histone-DNA interaction, is modified by O-GlcNAc, and found in a transcriptional repressor complex with mSin3A and OGT (51). Additionally, O-GlcNAcase is a histone acetyltransferase capable of relaxing histone structure (52, 53). Together these data suggests that OGT and O-GlcNAcase may affect DNA synthesis by altering chromosomal structure.

Even though DON is commonly used to lower O-GlcNAc levels in cells (11, 28, 38), it is a glutamine analogue and could potentially have side effects. DON-mediated reductions in the DNA synthesis and growth of neoplastic cell lines (54) are attributed to inhibition of de novo purine and pyrimidine biosynthesis (55). To exclude this possibility, our experiments were performed in media supplemented with 4 mM glutamine. Additionally, accelerated DNA synthesis is ablated upon incubation of double thymidine-blocked HeLa cells with glucosamine, which would circumvent DON inactivation of glutamine fructose-6-amidotransferase, suggesting the affects seen with DON can be partially attributed to changes in O-GlcNAc levels (Supplemental Fig. 1).

O-GlcNAc Levels Control the Expression of O-GlcNAcase and OGT—Cells maintain a steady state level of O-GlcNAc by controlling the protein expression of the O-GlcNAc-processing enzymes. PUGNAc-treated HeLa and 3T3-L1 cells caused a decrease in OGT levels and an increase in O-GlcNAcase levels, whereas DON treatment in 3T3-L1 cells caused a reciprocal change. In a cellular system finely tuned to react to changes in the environment, this feedback regulation either at the transcriptional or translational level is not surprising. Loss of the ability of the cells to maintain O-GlcNAc levels could lead to growth quiescence and apoptosis or contribute to the etiology of numerous metabolic diseases such as cancer, diabetes, and Alzheimer disease (9). For example, elevated O-GlcNAcase activity and decreased O-GlcNAc levels were found in human breast cancer tissue (56). Tau, a major component of paired-helical filamentous fibers in Alzheimer patients, is modified with O-GlcNAc. A significant loss in O-glycosylation is seen in post-mortem brains of Alzheimer...
O-GlcNAc Is a Dynamic Regulator of the Cell Cycle

disease patients, suggesting a loss of O-glycosylation concomitant with increased phosphorylation as the disease progresses (57, 58).

O-GlcNAcase and O-GlcNAc Transferase Control M-phase Phosphorylation—Overexpression of both vO-GlcNAcase and vOGT influences mitotic phosphorylation. The delay in maximal M phase phosphorylation after vO-GlcNAcase infection mirrors the flow cytometry data in which a large population of cells was delayed in exiting M phase compared with control. Similarly, the higher levels of mitotic phosphorylation after vOGT infection agree with the flow cytometry data showing that a large population of cells is in M phase after G1 release.

Phosphorylation of pRb, a specific protein involved in cell cycle regulation, is disrupted upon overexpression of vO-GlcNAcase and vOGT. Phosphorylation of pRb mirrored the proline-directed phosphorylation differences seen upon overexpression. This difference might be due to changes in specific protein–protein interactions between cell cycle proteins and vO-GlcNAcase/vOGT. These data strongly suggest that alterations in the expression of O-GlcNAcase or OGT lead to a mitotic exit phenotype. This phenotype could be caused by alterations in the expression of the cyclins.

O-GlcNAc as a Mediator of Cyclin Levels—As cells move through the cell cycle, the periodic expression of cyclins activates specific cyclin–dependent kinases, which in turn phosphorylate specific cell cycle substrates (2). The overexpression of both O-GlcNAcase and OGT disrupts proper cyclin periodicity. As HeLa cells are released at G1/S, the S-phase cyclin, cyclin E, is expressed. At release, vO-GlcNAcase-expressing cells have similar levels of cyclin E to control, but these levels decrease quicker than uninfected or control GFP cells. This corroborates the previous data in which decreased O-GlcNAc levels after DON treatment accelerated S phase. However, vO-GlcNAcase-expressing cells display reduced levels of cyclin E compared with control at the later G1 time points, suggesting errors in M phase release. The expression of vOGT severely disrupts cyclin E expression. These cells express low levels of cyclin E at the G1 release points and fail to re-express the cyclin at later time points, strengthening the argument for mitotic exit errors in these cells. The mitotic cyclins A and B show prolonged expression after PUGNAc treatment or vO-GlcNAcase and vOGT infection (Figs. 3 and 6). Again, these data strongly suggest delays or errors in proper M-phase progression in these cells. Furthermore, the disruption in cyclin expression could potentially lead to the aberrant phosphorylation seen in vO-GlcNAcase- and vOGT-expressing cells.

Control of the cyclin oscillator is through timed destruction of the appropriate cyclin by the proteasome (2), several components of which are modified by O-GlcNAc (59). Increased O-GlcNAc protein modification correlates with reduced proteasome function (60). In the case of vO-GlcNAcase overexpression, the prolonged expression of the cyclins is likely not due to the improper function of the proteasome since decreased O-GlcNAc does not inhibit the proteasome (60). However, this cannot be ruled out for vOGT overexpression and PUGNAc treatment.

Overexpression of OGT Interferes with Proper Cytokinesis—One potential mechanism for the increased number of cells in M-phase after vOGT overexpression is due to the inability of these cells to properly cytokinete. Notably, OGT co-localized with the central spindle assembly at M phase. Many MMP2-reactive epitopes are localized to the centromere along with several kinases such as POLO-like kinase and Aurora B (61, 62). The presence of OGT at the spindle assembly suggests that OGT acts in concert with mitotic kinases to delicately regulate cell division. OGT could dynamically add O-GlcNAc to specific proteins or act as a scaffold through its N-terminal TPR domain (63) to bring assembly proteins together. Several proline-directed phosphatases localize to the spindle assembly, such as CDC-14 (64), and control segregation of chromosomes by interacting with microtubule motor proteins (65). OGT is known to interact with protein phosphatase 1β and 1γ (66); therefore, OGT could act as an assembly protein recruiting phosphatases to the spindle assembly complex. Disruption of the interactions of OGT with components of the spindle assembly by overexpression of vOGT could lead to the increased number of cells in M phase. This is supported by the aberrant cellular morphology seen during cytokinesis.

Decision to divide and control the timing of these events is key to proper cellular function. O-GlcNAc, a metabolic nutrient and stress sensor, provides the cell with a mechanism to integrate multiple cellular signals to discriminatingly regulate cell cycle events. Coordinated regulation of the O-GlcNAc-processing enzymes would finely tune cells to environmental signals, allowing for proper cell cycle progression and controlled cytokinesis. Failure to properly regulate O-GlcNAc could then lead to cell cycle errors and diseases such as cancer and diabetes.

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O-GlcNAc Is a Dynamic Regulator of the Cell Cycle

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