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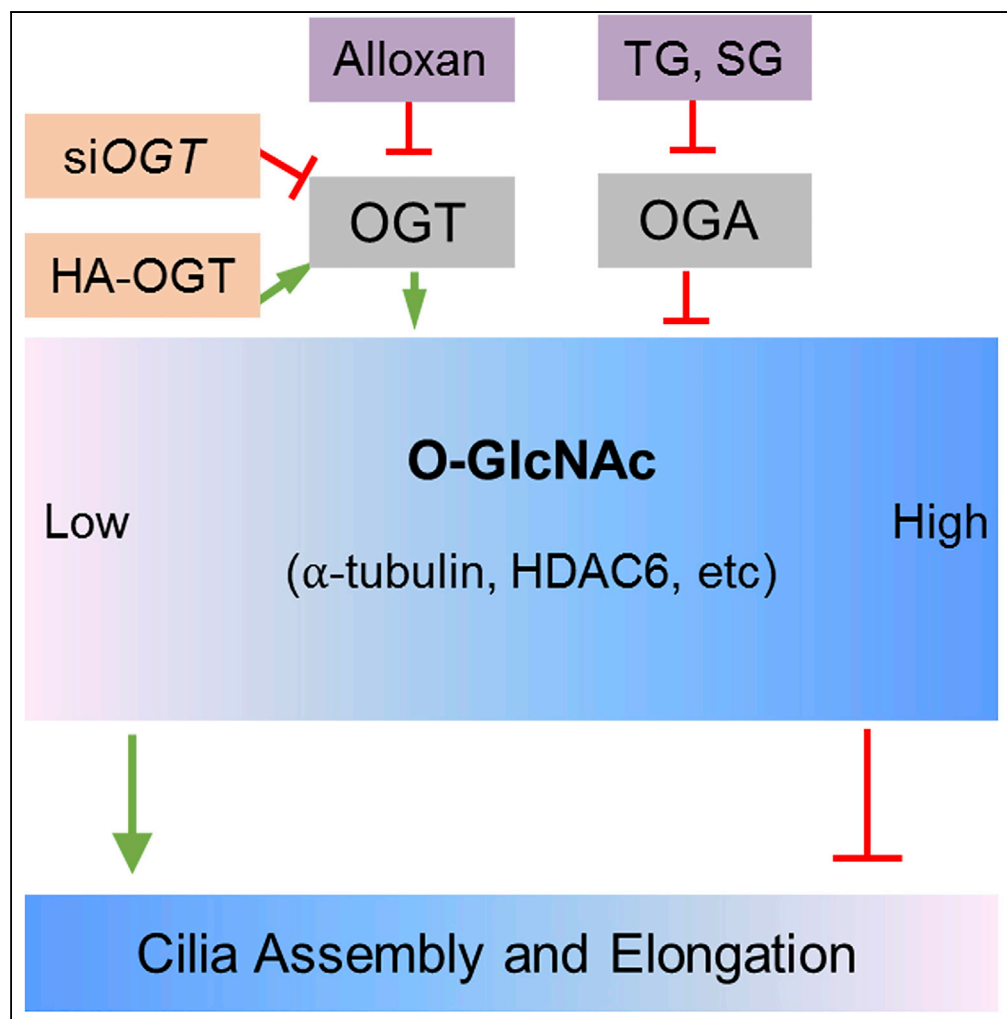
O-GlcNAcylation Regulates Primary Ciliary Length by Promoting Microtubule Disassembly

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HIGHLIGHTS
Cellular O-GlcNAc level inversely regulates ciliary length

Attenuated O-GlcNAc level increases the percentage of ciliated cells in hTERT-RPE1

O-GlcNAcylation of α-tubulin promotes ciliary axoneme disassembly

O-GlcNAcylation of HDAC6 promotes its deacetylase activity

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O-GlcNAcylation Regulates Primary Ciliary Length by Promoting Microtubule Disassembly

Jie L. Tian[1] and Hongmin Qin[1,2,3,*]

SUMMARY

The sensory organelle cilium is involved in sensing and transducing important signaling cascades in almost all cells of our body. These ciliary-mediated pathways affect cellular homeostasis and metabolisms profoundly. However, it is almost completely unknown whether the cellular metabolic state affects the assembly of cilia. This study is to investigate how O-linked β-N-acetylglucosamine (O-GlcNAc), a sensor of cellular nutrients, regulates the cilia length. Pharmacologic or genetic inhibition of O-GlcNAcylation led to longer cilia, and vice versa. Further biochemical assays revealed that both α-tubulin and HDAC6 (histone deacetylase 6) were O-GlcNAcylated *in vivo*. *In vitro* enzymatic assays showed that O-GlcNAcylation of either tubulin or HDAC6 promoted microtubule disassembly, which likely in turn caused ciliary shortening. Taken together, these results uncovered a negative regulatory role of O-GlcNAc in modulating the ciliary microtubule assembly. The cross talk between O-GlcNAc and cilium is likely critical for fine-tuning the cellular response to nutrients.

INTRODUCTION

Protein O-linked β-N-acetylglucosamine (O-GlcNAc) modification (O-GlcNAcylation) on serine and threonine residues is one of the most abundant metazoan nuclear-cytoplasmic post-translational modifications (PTMs). The addition and removal of O-GlcNAc are catalyzed by two enzymes O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), respectively (Bond and Hanover, 2015). UDP-GlcNAc, as a nucleotide sugar donor of enzyme OGT, is derived from the hexosamine biosynthetic pathway (HBP), which consumes 2%–5% of the total glucose (Marshall et al., 1991). The HBP metabolic pathway integrates multiple metabolites, such as carbohydrates, amino acids, fats, and nucleotides, to produce UDP-GlcNAc (Wells et al., 2003; Shi et al., 2012; Suizu et al., 2016). Therefore O-GlcNAc is sensitive to nutrient flux, and the abundance of O-GlcNAc reflects the internal cellular nutrient level. O-GlcNAc modulates basic biological functions and plays broad roles in multifaceted biological processes, such as gene expression (Zeidan et al., 2010; Hardiville and Hart, 2016), signal transduction (Vosseller et al., 2002; Yang et al., 2008), protein degradation (Ruan et al., 2013), cell cycle (Capotosti et al., 2011; Wang et al., 2010), autophagy (Wani et al., 2015; Guo et al., 2014), and cellular stress (Zachara et al., 2015). Perturbation of O-GlcNAcylation alters biological homeostasis, which is involved in a plethora of human pathologies, such as diabetes, obesity, neuron degenerative diseases, and cancer (Bond and Hanover, 2015).

O-GlcNAcylation regulates the activity of various components critical for metabolism and energy homeostasis, such as protein kinase B (AKT), glycogen synthase kinase-3β (GSK3β), hypoxia-inducible factor 1-alpha, and its transcriptional target glucose transporter. O-GlcNAcylation affects these proteins' activity or their stability (Ferrer et al., 2014; Shi et al., 2012). Interestingly, the above proteins are all either localized to the cilium or cilium-related structures or are essential for ciliogenesis (Suizu et al., 2016; Troilo et al., 2014; Yang et al., 2008; Zhang et al., 2015). The observations that these proteins either rely on the ciliary structure to function or are required for ciliary assembly intrigue us to ask whether there is a cross talk between O-GlcNAc and cilium.

Primary cilia are microtubule-based organelles enriched with receptors, channels, and signaling molecules (Wheway et al., 2018; Satir et al., 2010; Satir and Christensen, 2007). These ubiquitous structures, acting as "antenna" to transduce various extracellular signals, equip cells with diverse sensory functions. Many important signaling pathways, for instance, Hedgehog signaling pathway and Wnt signaling pathway, rely on primary cilia for proper function (Malicki and Johnson, 2017; Song et al., 2018). A wide range of ciliopathies arises due to defective cilia disrupting embryo development or tissue homeostasis (Eguether and Hahne, 2018; Guo et al., 2015; Hildebrandt et al., 2011).

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The backbone of cilia, axoneme, is a radial array of nine doublet microtubules with lengths normally ranging from 1 to 9 μm in humans (Dummer et al., 2016). Axonemal microtubules are highly stable and subjected to elaborate PTMs. Tubulin acetylation is the best studied PTM on ciliary axoneme (Janke and Bulinski, 2011; Ke and Yang, 2014). Acetylation of axonemal microtubules is catalyzed by α-tubulin acetyltransferase 1 (αTAT1), a conserved enzyme exclusively expressed in ciliated organisms and required for primary cilium assembly (Shida et al., 2010; Li et al., 2012). The αTAT1 activity is antagonized by histone deacetylase 6 (HDAC6), which is a tubulin deacetylase. Once activated, HDAC6 deacetylates acetylated axonemal microtubules. The deacetylated microtubules are unstable causing axonemal disassembly (Pugacheva et al., 2007). The deacetylase activity of HDAC6 is necessary and essential for ciliary microtubule disassembly (Ran et al., 2015; Pugacheva et al., 2007).

The tubulin deacetylase activity of HDAC6 is regulated by phosphorylation. Aurora kinase A (AurA) phosphorylates and actives HDAC6. The activated HDAC6 is then localized to the basal body and cilium by forming a complex with the scaffolding protein HEF1 (Pugacheva et al., 2007). The activation of AurA-HDAC6-HEF1 cascade is consistent with the two waves of primary cilia disassembly occurring post serum addition to G0 cells at 2 and 18 h, which correspond to G1 and mitosis phases, respectively. We reasoned that because O-GlcNAcylation and phosphorylation, the two prominent protein modifications, often tune protein's activity by competing for the same Ser and Thr residues or modifying the residues in defined patterns (van der Laarse et al., 2018), it is possible that the enzymatic activity of HDAC6 is regulated by O-GlcNAcylation as well. Indeed, in a prior liquid chromatography-mass spectrometry (MS) proteomic analysis, HDAC6 was found to be an O-GlcNAcylated protein (Gurel et al., 2014).

In this study, in cultured hTERT-RPE1 and IMCD3 cells, we demonstrate that the length of primary cilium negatively correlates with the cellular O-GlcNAc level. We also provide evidence that O-GlcNAcylation of either tubulin or HDAC6 promote axonemal microtubule disassembly. The cells appear to be able to attenuate the signaling response by limiting the size of cilium when nutrients are abundant. Clearly, the cross talk between O-GlcNAc and cilium puts the cell in a better position for fine-tuning the cellular response to nutrients.

RESULTS

Reduction of O-GlcNAc Caused Ciliary Length Elongation and Increased the Percentage of Ciliated Cells in Cultured hTERT-RPE1

OGT is the sole enzyme for addition of O-GlcNAc onto targeted proteins. To investigate the relationship between cilia and O-GlcNAc, we used small interfering RNA (siRNA) to knockdown the expression of the enzyme OGT in hTERT-RPE1 cells (Figure 1A). Immunoblotting (IB) of whole-cell lysates verified that the level of O-GlcNAcylated proteins was significantly reduced when OGT expression was knocked down by si*OGT* (Figure 1, A1), but remained expressed in cells transfected with a negative control siRNA to firefly luciferase GL2 (siGl2) (Figure 1, A1). Then we checked how reduction of protein O-GlcNAcylation affected the ciliary length and percentage of ciliated cells. Immunofluorescence (IF) staining with antibodies against ciliary marker proteins acetylated tubulin and IFT88 (Figure 1, A2) were used to highlight the ciliary structures. As the staining with anti-IFT88 marked the ciliary base and tip well, we used it to measure the ciliary length. In cells transfected with the negative control siGl2, 24.18% ± 6.8% of cells were ciliated. In contrast, in cells transfected with si*OGT*, the percentage of ciliated cells was increased to 55.38% ± 8.8% (Figure 1, A3). In addition, the mean ciliary length of cells treated with si*OGT* was 5.07 ± 0.012 μm, which was significantly longer than that of the cells treated with siGl2 (3.62 ± 0.322 μm) (Figure 1, A4). Therefore a lower cellular level of O-GlcNAcylation positively affected ciliary assembly, promoting both cilia formation and elongation.

To verify that the effects seen in si*OGT*-treated cells were indeed caused by the reduction of protein O-GlcNAcylation, an si*OGT*-resistant OGT (HA-OGT-rescue) (Li et al., 2017) was expressed in si*OGT* cells (Figures 1, B1 and 1B2). Based on the band intensity on immunoblots of whole-cell lysates, the expression of the endogenous OGT was decreased ~75% in cells treated with si*OGT*, but not in cells transfected with siGl2. The si*OGT*-resistant HA-OGT-rescue, however, was successfully expressed when endogenous OGT was silenced, as shown by the IB with an antibody against HA (Figure 1, B1). We then checked whether the expression of HA-OGT in si*OGT* knockdown cells shortened the ciliary length or not (Figure 1, B2 and 1B3). Indeed, expression of HA-OGT in OGT-depleted si*OGT*-treated cells restored the ciliary length to normal. The mean ciliary length of cells expressing HA-OGT-rescue was 3.9 ± 0.36 μm, which was statistically
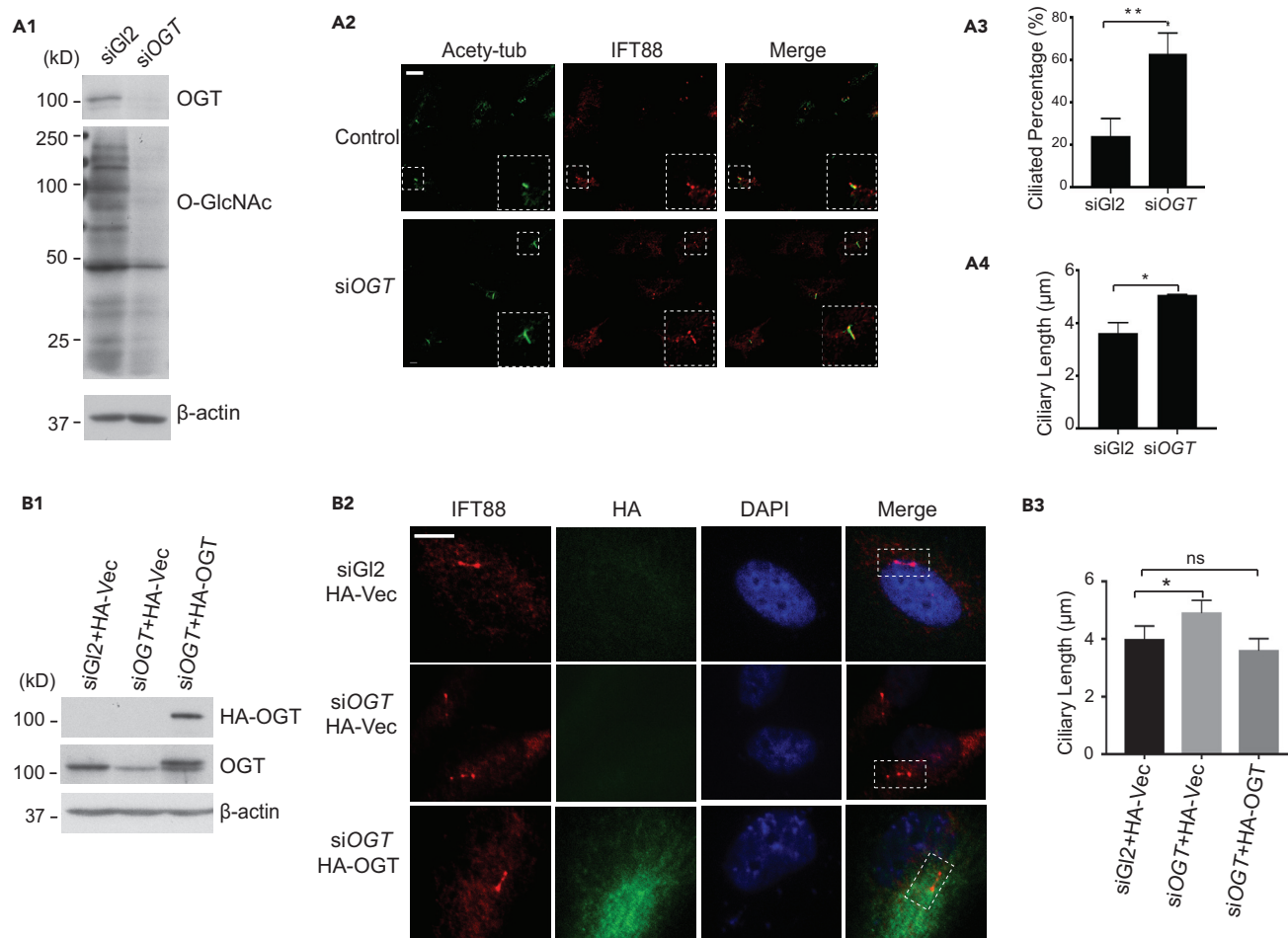
Figure 1. Cilium Length and Percentage of Ciliated Cells Were Increased when O-GlcNAc Level Was Reduced in hTERT-RPE1 Cells

(A) si*OGT*-transfected cells were used to check the effects of O-GlcNAc reduction on ciliary length and the percentage of ciliated cells. siGl2 was the negative control. Three independent replicates were performed. Representative results are shown. (A1) Immunoblots of cell lysates with indicated antibodies. (A2) Confocal images of cells co-immunostained with antibodies against acetylated tubulin and IFT88. (A3) Bars represent the percentage of cells with primary cilia in siGl2 and si*OGT* cells (**$p < 0.01$ [$p = 0.006$]). (A4) Mean lengths of cilia in siGl2 and si*OGT* knockdown cells (*$p < 0.05$ [$p = 0.0126$]). (B) Expression of HA-OGT-rescue in si*OGT* knockdown cells restored ciliary length to normal. (B1) IB analysis of endogenous OGT and exogenous HA-OGT-rescue expressions. (B2) Confocal images of cells co-immunostained with antibodies against IFT88 and HA. (B3) Mean length of cilia in groups as indicated on the graph (*$p < 0.05$ [$p_{siOGT/HA-Vec} = 0.044$, $p_{siOGT/HA-OGT} = 0.33$]; ns, not significant). β-actin was used to show equal protein loadings on immunoblots. Scale bars, 5 μm. All data are mean ± SD.

shorter than that of the si*OGT* cells (4.99 ± 0.31 μm). These results confirmed that protein O-GlcNAcylation catalyzed by OGT was responsible for the elongated cilia seen in si*OGT* cells.

The O-GlcNAcylation-Mediated Ciliary Length Regulation Was Not hTERT-RPE1 Specific, but Was Also Seen in IMCD3 Cells

To evaluate whether the effect of O-GlcNAcylation on ciliary length was cell line specific, we examined whether changes of OGT expression regulated length of cilia in another cell line, IMCD3. Consistent with the results seen in hTERT-RPE1 cells (Figure 1A), the IMCD3 cells assembled longer cilia when protein O-GlcNAcylation was inhibited by si*OGT* (Figures 2A1–2A3). We did not detect a significant change of the percentage of ciliated cells when OGT expression was inhibited. The percentage of ciliated cells remained at about 40%.

We reasoned that as O-GlcNAcylation inhibition induced ciliary elongation, it is possible that an elevated O-GlcNAcylation would lead to shorter cilia. To test this idea, we transiently overexpressed an HA-tagged
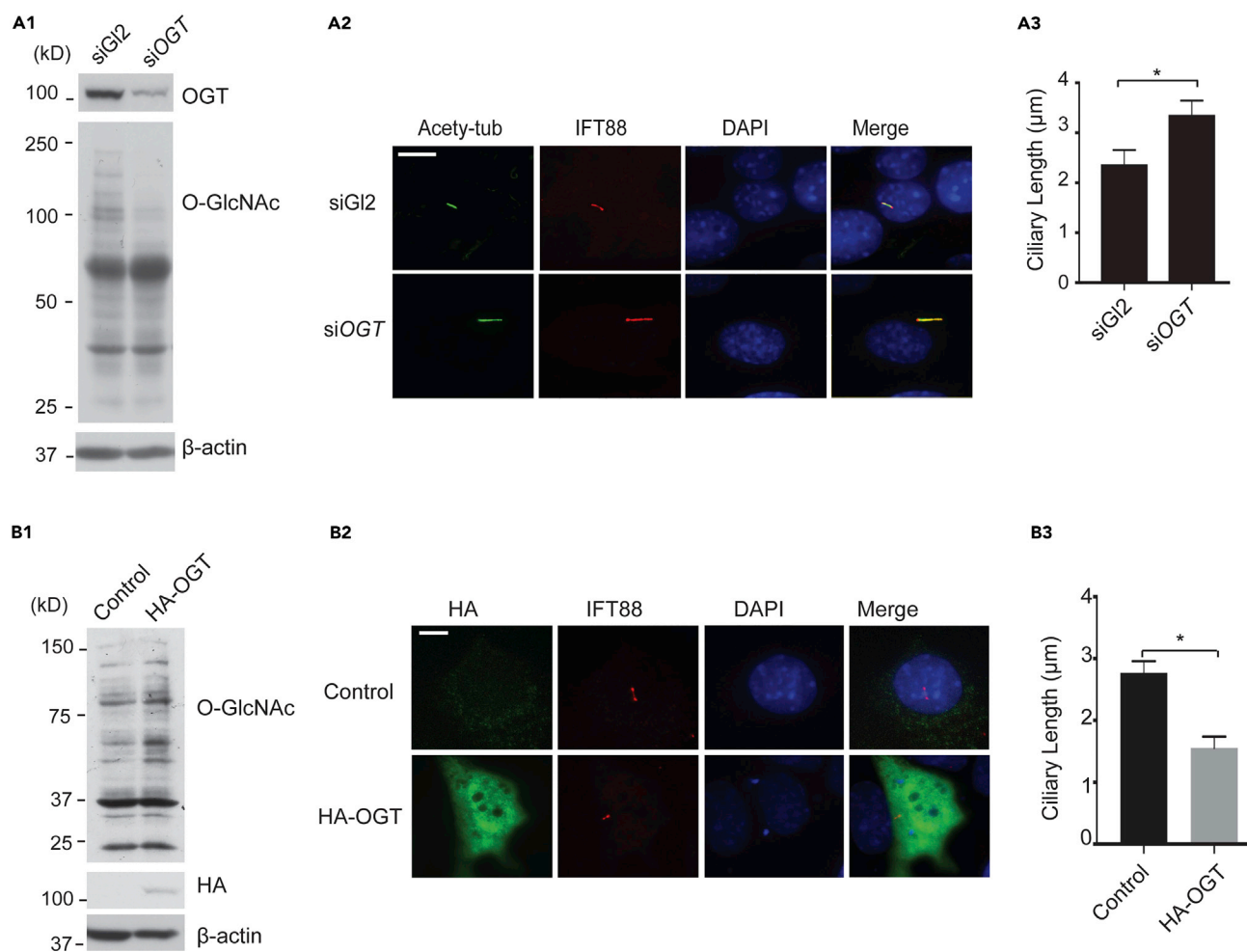
Figure 2. Knockdown of OGT Increased Ciliary Length, whereas HA-OGT Overexpression Decreased Ciliary Length in IMCD3

(A) Cells transfected with si*OGT* or the negative control siGl2 were used for analysis. Three independent replicates were performed. Representative results are shown. (A1) IB analysis of O-GlcNAc levels in siGl2- and si*OGT*-transfected cells with indicated antibodies. (A2) Confocal images of cells co-immunostained with antibodies against acetylated tubulin and IFT88. (A3) The si*OGT* knockdown cells had longer cilia (*p < 0.05 [p = 0.0126]).

(B) Overexpression of HA-OGT decreased ciliary length. (B1) IB analysis of O-GlcNAc levels in HA-vector and HA-OGT-overexpressed cells. (B2) Confocal images of cells co-immunostained with antibodies against acetylated tubulin and IFT88. (B3) The ciliary length was shorter when cells overexpressed HA-OGT (*p < 0.05 [p = 0.017]).

Scale bars, 5 μm. All data are mean ± SD.

OGT in IMCD3 cells. The increased protein O-GlcNAcylation in HA-OGT-overexpressed cells was verified by IB (Figure 2, B1). The mean length of HA-OGT-positive cells was 1.55 ± 0.18 μm, which was significantly shorter than that of control cells (2.61 ± 0.4 μm) (Figures 2B2 and 2B3). Taken together, in both hTERT-RPE1 and IMCD3 cells, there is a negative correlation between O-GlcNAcylation and ciliary length: the higher cellular O-GlcNAcylation, the shorter the ciliary length.

Inhibition of OGT or OGA by Small Chemical Inhibitors Confirmed the Negative Regulatory Role of O-GlcNAcylation on Ciliary Length

We selected three specific small chemical inhibitors: Thiamet G (TG) (Ding et al., 2014) and GlcNAcstatin G (SG) for OGA (Dorfmueller et al., 2006; Sousa et al., 2013) and alloxan (Alxn) for OGT (Konrad et al., 2002), to treat both IMCD3 and hTERT-RPE1 cells. The efficiency of these inhibitors on protein O-GlcNAcylation was first confirmed by IB against an anti-O-GlcNAc antibody (Figures 3A1 and 3B1). Consistent with prior published results, Alxn treatment decreased overall protein O-GlcNAcylation, whereas TG or SG treatment increased cellular O-GlcNAcylation. Then the length of cilia was measured using IF staining of ciliary marker

A1

A2

A3

B1

B2

B3
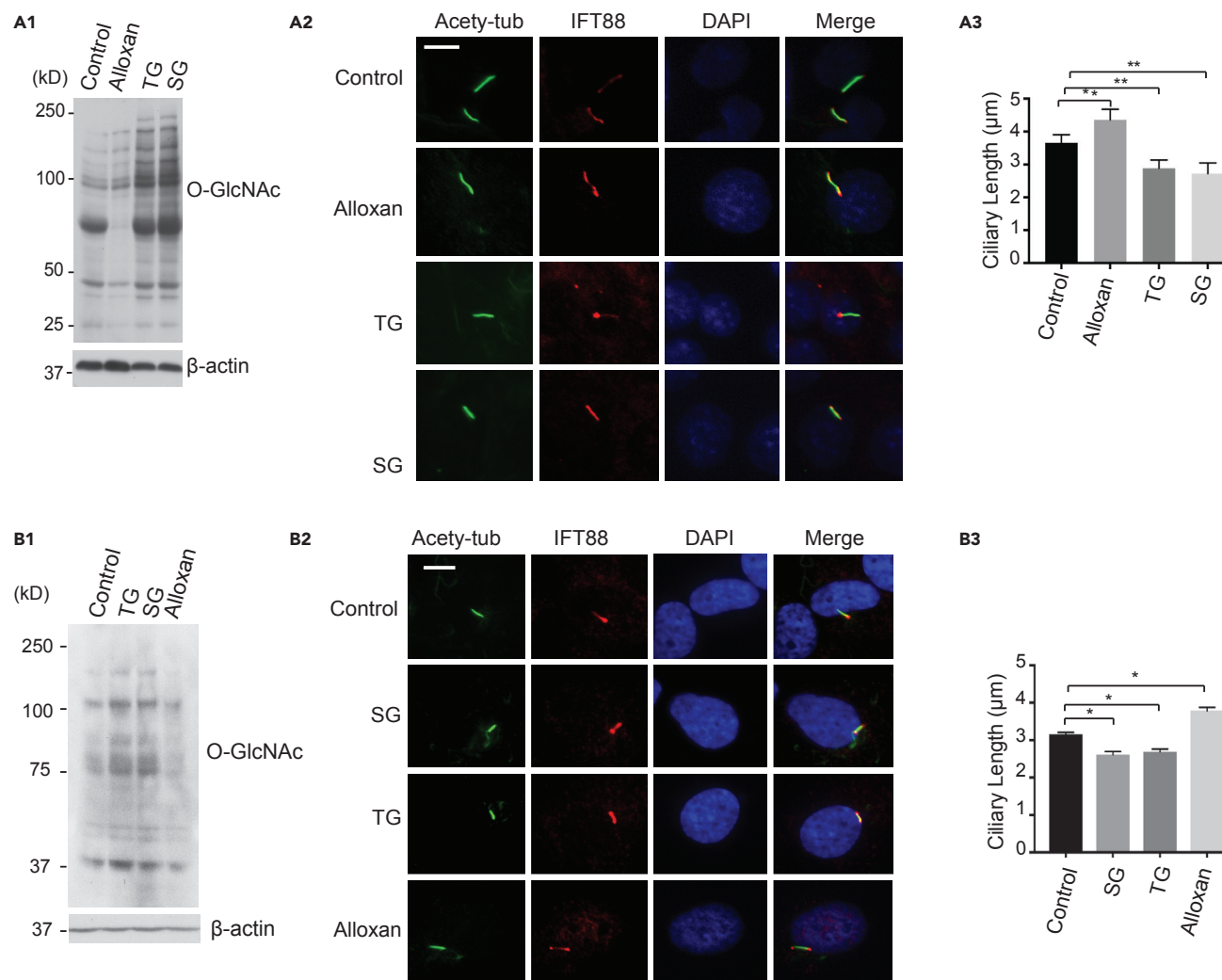
Figure 3. The Negative Correlation between O-GlcNAcylation Level and Cilia Length Verified by Treating Cells with OGT and OGA Inhibitors

The IMCD3 (A) and hTERT-RPE1 (B) cells were subjected to OGT inhibitor alloxan, OGA inhibitor TG or SG, or control DMSO (A1 and B1). IB analysis of O-GlcNAc levels in treated cells. β-actin was used to show equal protein loadings. (A2 and B2) Confocal images of cells co-immunostained with antibodies against acetylated tubulin and IFT88. Scale bars, 5 μm. (A3 and B3) The graph shows the statistical analysis of ciliary lengths of treated groups (*p < 0.05, **p < 0.01, A3: pAlloxan = 0.009, pTG = 0.0014, pSG = 0.004; B3: pSG = 0.018, pTG = 0.016, $p_{Alloxan}$ = 0.039). All data are mean ± SD.

protein IFT88. The length of cilia in either IMCD3 or hTERT-RPE1 was longer with Alxn treatment. The mean length of Alxn-treated IMCD3 cells was 4.36 ± 0.32 μm, which was significantly longer than that of the control group (4.0 ± 0.37 μm). The mean ciliary length of Alxn-treated hTERT-RPE1 cells was 3.79 ± 0.09 μm, whereas the length of control cells was 3.26 ± 0.2 μm. Conversely, the ciliary lengths were significantly shorter in cells treated with TG or SG. The mean length was shortened to 2.88 ± 0.24 μm with TG treatment, and to 2.72 ± 0.32 μm with SG treatment in IMCD3 cells. The TG and SG treatments caused the ciliary lengths of hTERT-RPE1 cells to shorten to 2.73 ± 0.11μm and 2.64 ± 0.09 μm, respectively (Figures 3A2, 3A3, 3B2, and 3B3). These small chemical treatment results (Figure 3) were consistent with the observations obtained by perturbing the expression of OGT and OGA (Figures 1 and 2).

O-GlcNAcylation of α-Tubulin Promoted Ciliary Axoneme Disassembly

The ciliary length is determined by the rate of microtubule assembly and disassembly in the axoneme. To understand how O-GlcNAcylation affects ciliary length, we asked which proteins, once O-GlcNAcylated,
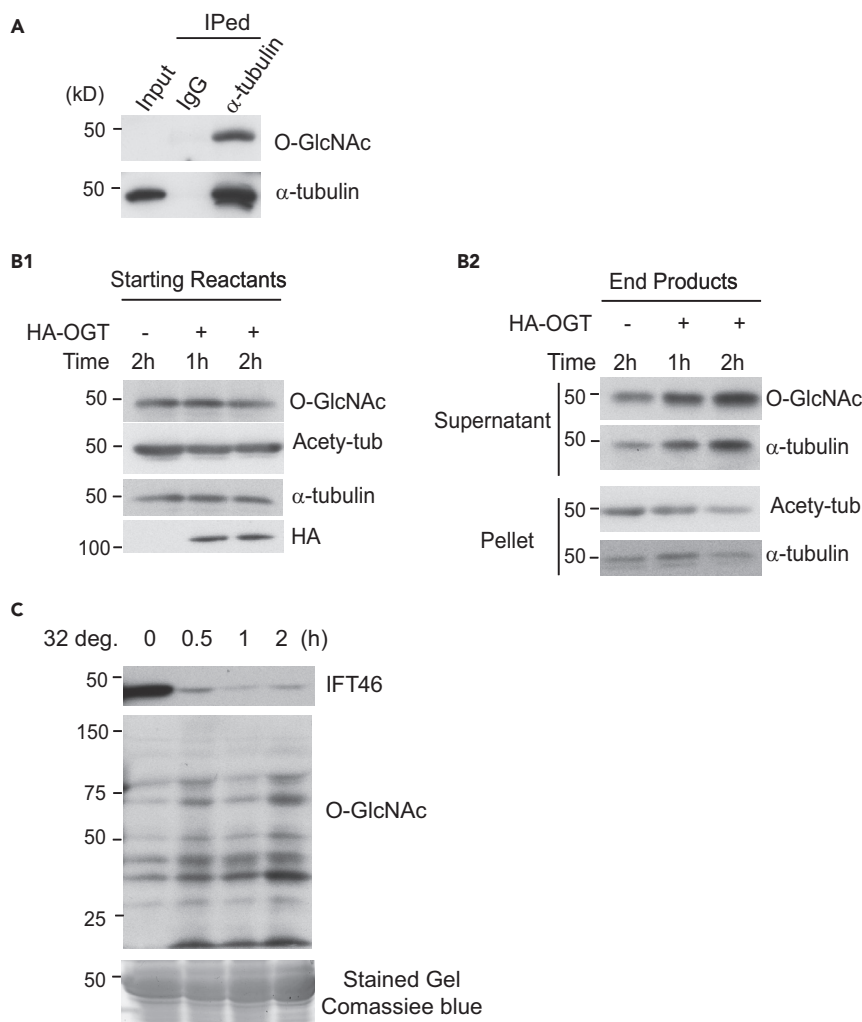
Figure 4. O-GlcNAc of α-Tubulin Related to Instability of Flagellar Axoneme

(A) Endogenous α-tubulin was IPed and subjected to IB with anti-α-tubulin and anti-O-GlcNAc antibodies.

(B1 and B2) (B1) Purified HA-OGT was incubated with flagellar axonemes for 1 or 2 h. IB analysis showed the relative amounts of input reactants of HA-OGT (probed with anti-HA), axonemes (blotted with antibodies against α-tubulin and acetylated-tubulin), and the base level of O-GlcNAc modification of axonemal microtubules (labeled as O-GlcNAc). (B2) After 1 or 2 h, the supernatants and pellets were separated for IB analysis.

(C) Flagella of $fla10^{ts}$ harvested after cells were incubated at 32°C for 0, 0.5, 1, and 2 h were used for IB analysis. The levels of O-GlcNAc were increased in $fla10^{ts}$ flagella from cells incubated at 32°C. The disappearance of IFT46 was used to verify that flagella were undergoing disassembly.

could regulate axonemal microtubule dynamics. A previous study showed that O-GlcNAcylated α-tubulin fails to incorporate into microtubules (Ji et al., 2011), suggesting an inhibitory role of tubulin O-GlcNAcylation in microtubule assembly. Using immunopurified endogenous α-tubulin, we confirmed that α-tubulin was O-GlcNAcylated in hTERT-RPE1 cells (Figure 4A). We then used a direct *in vitro* O-GlcNAcylation assay to examine how tubulin O-GlcNAcylation affects the stability of axonemal microtubules. HA-OGT was overexpressed in hTERT-RPE1 cells for 48 h and then purified with anti-HA antibody attached to protein A beads. We then checked if the purified HA-OGT could catalyze O-GlcNAcylation of axonemal microtubules *in vitro*, and if O-GlcNAcylation promoted axonemal microtubule disassembly (Figure 4B). The results showed that the amount of O-GlcNAcylated α-tubulin increased in reactions in which HA-OGT was added, indicating that α-tubulin is a substrate of OGT. Moreover, the amount of free α-tubulin in the supernatants was increased compared with the reaction without HA-OGT. The

supernatant contained a higher amount of disassembled free tubulin in reaction prolonged for 2 h than in the one prolonged for just 1 h (Figure 4B2). This result indicated that O-GlcNAcylation of α-tubulin promoted disassociation of tubulin from axonemal microtubules.

We reasoned that because O-GlcNAcylation promoted axonemal microtubule disassembly, cilia undergoing disassembly were likely to have a higher level of O-GlcNAcylation. To test this prediction, we turned to a temperature-sensitive flagellar mutant $fla10^{ts}$ of the green algae *Chlamydomonas reinhardtii*. The mutant $fla10^{ts}$ harbors a point mutation in the kinesin-II motor subunit FLA10 and is functionally normal in flagellar assembly at the permissive temperature (18°C) but abolishes the anterograde intraflagellar transport (IFT) at the non-permissive temperature (32°C). The inactivation of IFT at 32°C induces flagellar axonemal disassembly (Cole et al., 1998; Walther et al., 1994). We checked the levels of protein O-GlcNAcylation in $fla10^{ts}$ flagella that were undergoing disassembly. The results showed that O-GlcNAcylation was increased in disassembly-active flagella (Figure 4C).

O-GlcNAcylation of HDAC6 Is Involved in Ciliary Length Shortening

HDAC6 plays an important role in cilia assembly. Recent proteomic analysis shows that HDAC6 is an O-GlcNAcyated protein (Gurel et al., 2014). It is possible that O-GlcNAcylation activates HDAC6 deacetylase activity, which leads to cilia shortening. If this is true, we reasoned that ciliary shortening induced by elevated protein O-GlcNAcylation should be blocked by the inhibition of HDAC6. To test this idea, the hTERT-RPE1 cells were treated with HDAC6 inhibitor tubacin (Haggarty et al., 2003) or together with the OGA inhibitor TG or SG (Figure 5). The ciliary length analysis showed that inhibition of HDAC6 activity indeed prevented cells from shortening caused by TG or SG treatment (Figure 5B). This result suggested that the elevated HDAC6 deacetylase contributed to the shorter cilia induced by high O-GlcNAcylation.

To examine the underlying mechanisms of how O-GlcNAcylation affected HDAC6, we first checked whether HDAC6 and OGT interacted *in vivo*. Immunoprecipitation using either anti-HDAC6 or anti-OGT antibody confirmed that these two proteins could be co-immunoprecipitated (co-IPed) reciprocally from lysates of hTERT-RPE1 cells (Figure 6A). The IPed HDAC6 was O-GlcNAcylated (Figure 6B), confirming the prior MS proteomic data (Gurel et al., 2014). Collectively, these results showed that HDAC6 was a substrate of OGT.

O-GlcNAcylation could potentially increase the HDAC6 deacetylase activity by two means: increasing the protein amount or increasing the actual enzymatic activity. We confirmed that the amount of HDAC6 protein was higher when O-GlcNAcylation was promoted by OGT overexpression (Figure 6C) or by OGA inhibition (Figure 6D). We then checked whether O-GlcNAcylation of HDAC6 affected its deacetylase activity (Figure 6E). The HDAC6 protein was IPed from the lysates of hTERT-RPE1 cells treated with either TG or SG. Equal amounts of purified HDAC6 protein from cells with various treatments were incubated with axonemal microtubules purified from *Chlamydomonas* for 1 h at 37°C. HDAC6 from cells treated with TG or SG had a higher tubulin deacetylation activity compared with the protein from control cells. This result hinted that O-GlcNAc modification of HDAC6 enhanced its deacetylase activity. Taken together, the increased activity of HDAC6 in cells with high O-GlcNAc levels may come from changes of its protein amount as well as from increased enzymatic activity.

O-GlcNAcylation of INPP5E Did Not Affect Its Ciliary Localization in hTERT-RPE1

Inositol polyphosphate 5-phosphatase (INPP5E) is a ciliopathy disease protein involved in Joubert and mental retardation, truncal obesity, retinal dystrophy, and micropenis (MORM) syndromes (Travaglini et al., 2013; Jacoby et al., 2009). INPP5E plays an important role in the stability of primary cilia by affecting the ciliary membrane lipid composition (Bielas et al., 2009; Garcia-Gonzalo et al., 2015). Moreover, both HDAC6 and INPP5E are substrates of AurA kinase (Plotnikova et al., 2015). We asked whether INPP5E is O-GlcNAcylated and whether its function is affected by O-GlaNAcylation. We first checked whether INPP5E was a substrate of OGT (Figure S1). INPP5E and OGT could be co-IPed reciprocally. INPP5E was O-GlcNAcylated, confirming that it was a substrate of OGT (Figures S1A and S1B). However, the amount of O-GlcNAcylated INPP5E did not change even when the overall O-GlcNAcylated protein amount increased with OGA inhibitor treatments (Figure S1C). Moreover, the ciliary localization of INPP5E appeared to be normal in either OGT loss- or gain-of-function cells (Figures S1D and S1E).

A

B
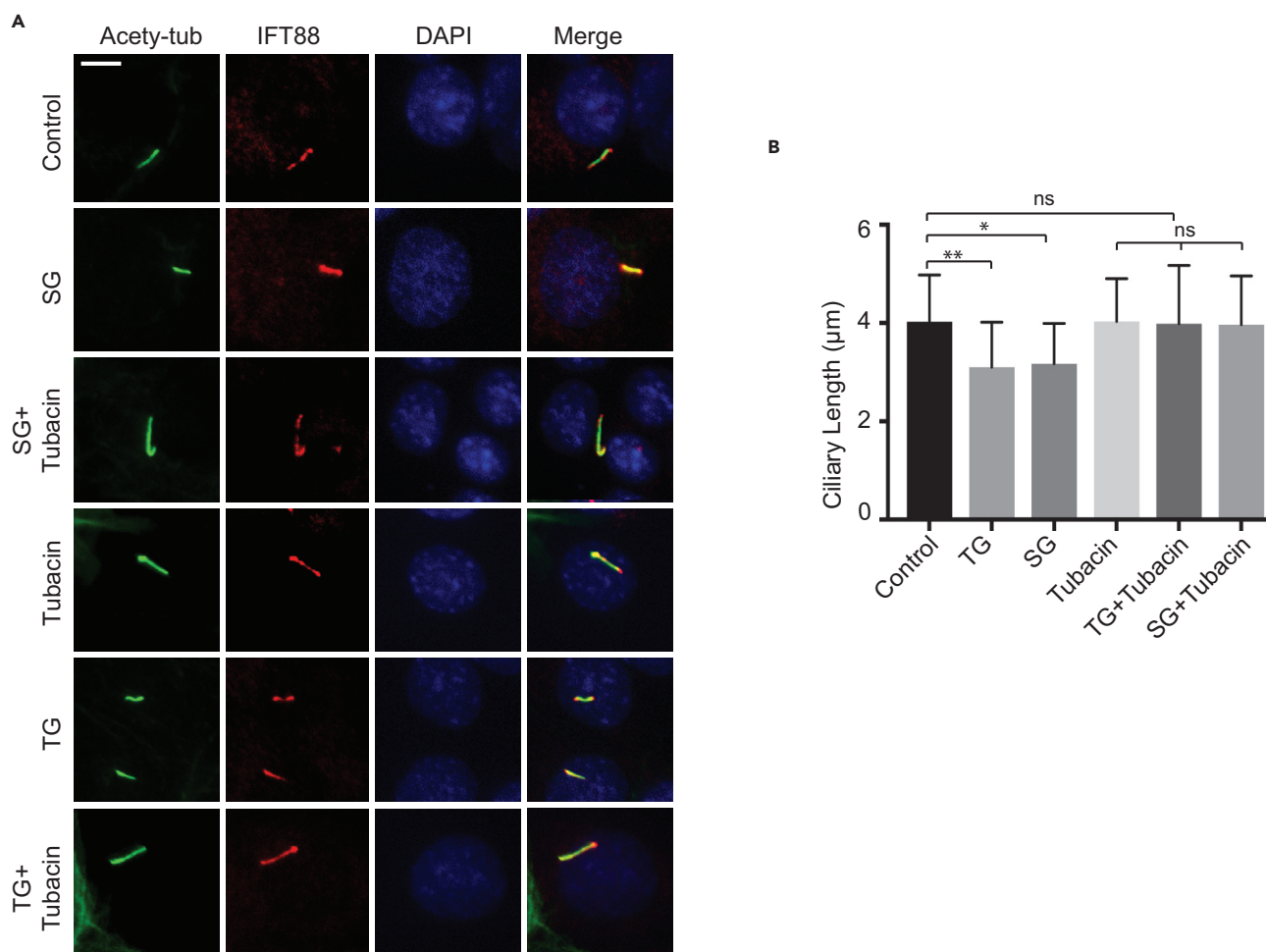
Figure 5. Inhibition of HDAC6 Suppressed Ciliary Resorption Induced by TG or SG Treatment

The hTERT-RPE1 cells were subjected to TG, SG, TG + tubacin, SG + tubacin, or control DMSO treatment.

(A) Confocal images of cells co-immunostained with antibodies against acetylated tubulin and IFT88. Scale bar, 5 μm.

(B) The mean length of cilia of cells with different treatments (*p < 0.05, **p < 0.01 [p_{TG} = 0.001, p_{SG} = 0.017, $p_{Tubacin}$ = 0.97, $p_{TG/Tubacin}$ = 0.8, $p_{SG/Tubacin}$ = 0.74]). Data are mean ± SD.

DISCUSSION

This study uncovered a negative regulatory role of O-GlcNAcylation on primary ciliary length in both hTERT-RPE1 and IMCD3 cells. In OGT gain-of-function cells, ciliary length was shortened, whereas ciliary length was elongated in OGT loss-of-function cells. O-GlcNAcylation of α-tubulin promoted its disassociation from the axoneme, which might promote the disassembly of axoneme. O-GlcNAcylation of HDAC6 activated its deacetylase activity to deacetylate axonemal microtubule, which was likely causing cilia to resorb. Based on these results, we concluded that the ciliary length is sensitive to the cellular level of O-GlcNAc. We propose that the constant cross talk between the primary cilium and O-GlcNAc is an important mechanism for cells fine-tuning the cellular responses to nutrients (Figure 7).

This study brings O-GlcNAc as an important input signal to ciliary assembly and function. Primary cilia act as a cellular "antenna" that receives diverse signals from the extracellular environment, including chemical and mechanical stimuli. The past studies of a wide spectrum of human ciliopathies, for example, polycystic kidney disease, Bardet-Biedl syndrome, and orofaciodigital syndrome (Malicki and Johnson, 2017; Hildebrandt et al., 2011), demonstrate that the cilium is an essential organelle for almost every aspect of development and tissue homeostasis. The negative feedback regulation of ciliary length by the key nutrient sensor O-GlcNAc shown by this study appears to be a mechanism of sensory signal adaptation. The
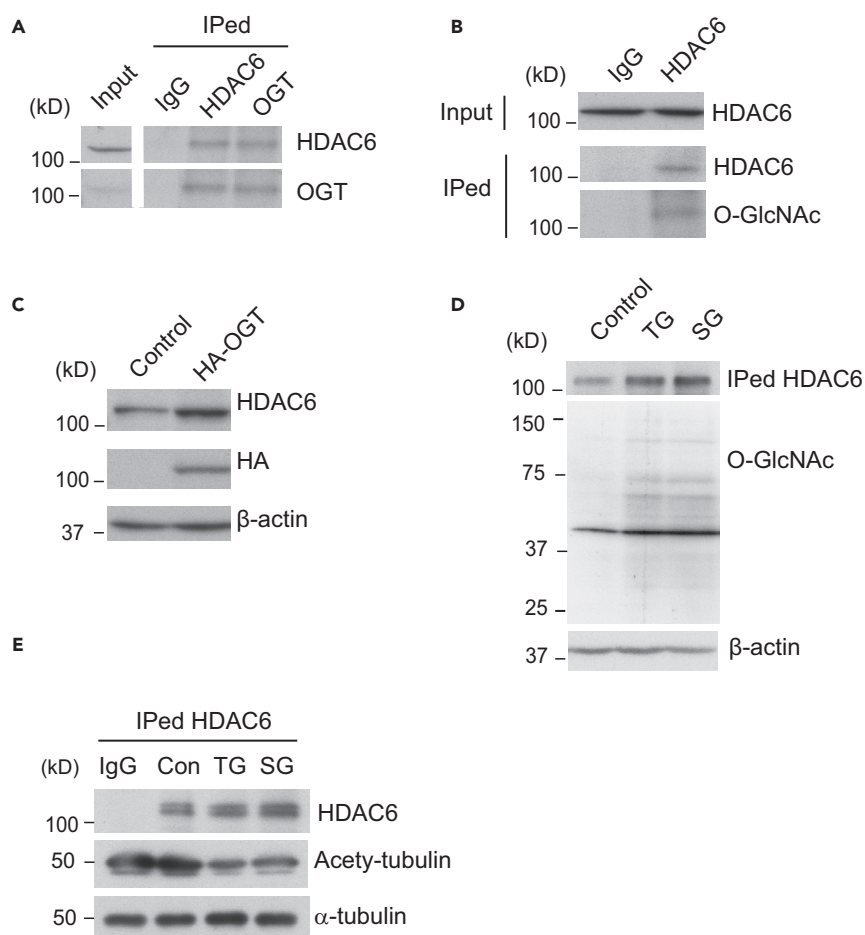
Figure 6. HDAC6 Was O-GlcNAcylated, and O-GlcNAc Modification of HDAC6 Enhanced its Deacetylase Activity

(A) Protein OGT and HDAC6 were reciprocally co-IPed together from cell lysates. IB analysis of HDAC6 and OGT in immunoprecipitates using anti-HDAC6, anti-OGT, or control rabbit IgG.

(B) HDAC6 protein was O-GlcNAcylated. HDAC6 protein was IPed from cell lysates and then subjected to IB with antibodies against HDAC6 and O-GlcNAc.

(C) The amount of HDAC6 level in HA-OGT overexpression cells was increased.

(D) The amount of HDAC6 IPed using the same amount of anti-HDAC6 antibody from TG or SG-treated cell lysates was higher than that from the control treated with DMSO (Con) lysates.

(E) The same amount of immunopurified HDAC6 proteins from TG or SG treated, or DMSO (control) cell lysates were used in tubulin deacetylase (TDAC) assays. After reactions were ended, the samples were subjected to IB to check the levels of indicated proteins. The levels of acetylated-tubulin were reduced in samples containing HDAC6 purified form TG- or SG-treated cell lysates.

elongation of the cilium is to increase its signal detection when nutrients are scarce, whereas the resorption is to limit its responsiveness to external signals. By adjusting their size depending on the abundance of nutrients, cilia allow cells to tune out random nutrient flux fluctuations and to up- or downregulate cilia sensitivity to nutritive environment. Obviously, the constant dialog between cilia and O-GlcNAc is beneficial to maintain O-GlcNAc homeostasis, which is critical to many cellular processes, including cell cycle progress, stress response, and gene transcription (Zhang et al., 2014). Disruptions in O-GlcNAc homeostasis are proposed to lead to the development of diseases, such as cancer, diabetes, and Alzheimer disease (Shi et al., 2018; Zhang et al., 2014; de Queiroz et al., 2016; Bond and Hanover, 2015; Qian et al., 2018).

A wide range of signals from cell cycle, protein synthesis, autophagy, and cytoskeletons not only rely on cilia to function but also regulate the assembly and resorption of cilia (Malicki and Johnson, 2017). For example, these pathways modulate the activity of HDAC6 (Ran et al., 2015), OFD1 (Tang et al., 2013), mammalian target of rapamycin (Boehlke et al., 2010), GSK3β (Thoma et al., 2007), or other enzymes and
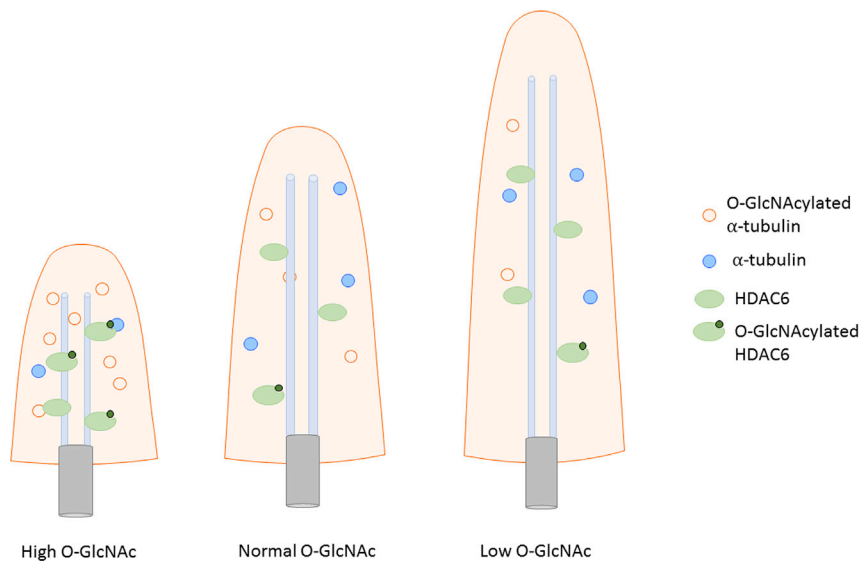
Figure 7. Molecular Model of O-GlcNAc in Regulating Ciliary Assembly and Disassembly

When the cellular O-GlcNAc level is high, more axonemal α-tubulins are O-GlcNAcylated. These tubulins disassociate from axonemal microtubules, which promotes axonemal disassembly. O-GlcNAcylation of HDAC6 also enhances its deacetylase activity, which leads to axonemal disassembly. Collectively, the ciliary length is shorter at a high O-GlcNAc cellular condition. Conversely, a low O-GlcNAc level stabilizes axonemal microtubules, which allows longer cilia to assemble. Therefore the cross talk at the molecular level between O-GlcNAc and cilium puts the cell in a better position for fine-tuning the cellular response to nutrients.

regulatory proteins that are important for the ciliogenesis (Keeling et al., 2016). However, very little is known about which of the signaling proteins or messengers integrates these diverse signals or whether there is a hierarchical order of these signal inputs on cilium length regulation. In this study, we showed that the level of protein O-GlcNAcylation negatively regulates the length of cilia. As O-GlcNAcylation integrates multiple metabolites, such as carbohydrates, amino acids, fats, and nucleotides, it is well suited to tell the nutritive state of an organism. Future research is required to examine whether several ciliary signal inputs converge on O-GlcNAc to regulate ciliary length.

Biochemical analysis of isolated flagella from *Chlamydomonas* showed that many flagellar axonemal proteins were O-GlcNAcylated (Figure 4C). Moreover, the overall O-GlcNAc level was much higher in flagella undergoing disassembly (Figure 4C), supporting that upregulated O-GlcNAc leads to ciliary resorption. In this study we checked a couple of proteins with well-established functions in ciliogenesis on how their O-GlcNAcylation affects axonemal microtubules. Although INPP5E was O-GlcNAcylated, we failed to link this modification to its ciliary function (Figure S1). We found that O-GlcNAcylation of α-tubulin promoted its disassociation from axonemal microtubules (Figure 4B). The axonemal microtubules are heavily post-translationally modified with acetylation, glycylation, and glutamylation (Yu et al., 2015). Tubulin acetylation and glycylation stabilize axonemal microtubules. In contrast, polyglutamylation plays an opposite role, destabilizing axonemal microtubule (Kubo et al., 2015; Wloga et al., 2017). Here, we showed that O-GlcNAcylation is another microtubule (MT) destabilizer. Balanced tubulin PTMs are important for ciliary length regulation. Future research on whether O-GlcNAcylation of tubulin coordinates with other tubulin PTMs will be of particular interest to cilia length regulation and ciliogenesis.

In this study, we showed that HDAC6 was subjected to O-GlcNAcylation and O-GlcNAc modification of HDAC6 promoted its enzymatic activity. HDAC6 is a key regulator of tubulin acetylation and functions in regulating MT stability (Ran et al., 2015). The activation of HDAC6 involves its phosphorylation by AurA kinase. Once activated, HDAC6 deacetylates acetylated MTs, leading to axonemal resorption. The mechanism of how O-GlcNAcylation of HDAC6 enhances its tubulin deacetylase activity is currently unknown. As interplays between phosphorylation and O-GlcNAcylation are common, future research will be able to test whether O-GlcNAcylation of HDAC6 has a synergic effect on its phosphorylation, which in turn upregulates its activity.

Limitations of the Study

In this study hTERT-RPE1 and IMCD3 cell lines were used to investigate the regulatory roles of O-GlcNA-cylation on primary ciliary length. Future work should include animal models. Although *in vitro* biochemistry analysis linked O-GlcNAcylation of α-tubulin and HDAC6 to primary ciliary length control, subsequent studies are necessary to elucidate whether O-GlcNAc of α-tubulin and HDAC6 affect MT dynamics *in vivo*. This study also leaves the question how HDAC6 deacetylase activity is promoted by increased cellular O-GlcNAcylation levels unanswered. Could it be due to the interplay between O-GlcNAcylation and phosphorylation, or changes in the protein level of HDAC6? Moreover, it is worth noting that many ciliary proteins are O-GlcNAcylated. This study is no more than the tip of the iceberg of O-GlcNAc on ciliary signaling and assembly.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Transparent Methods, one figure, and one table and can be found with this article online at https://doi.org/10.1016/j.isci.2019.01.031.

AUTHOR CONTRIBUTIONS

J.L.T. designed and performed the experiments; J.L.T. and H.Q. analyzed the data and wrote the manuscript; H.Q. supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

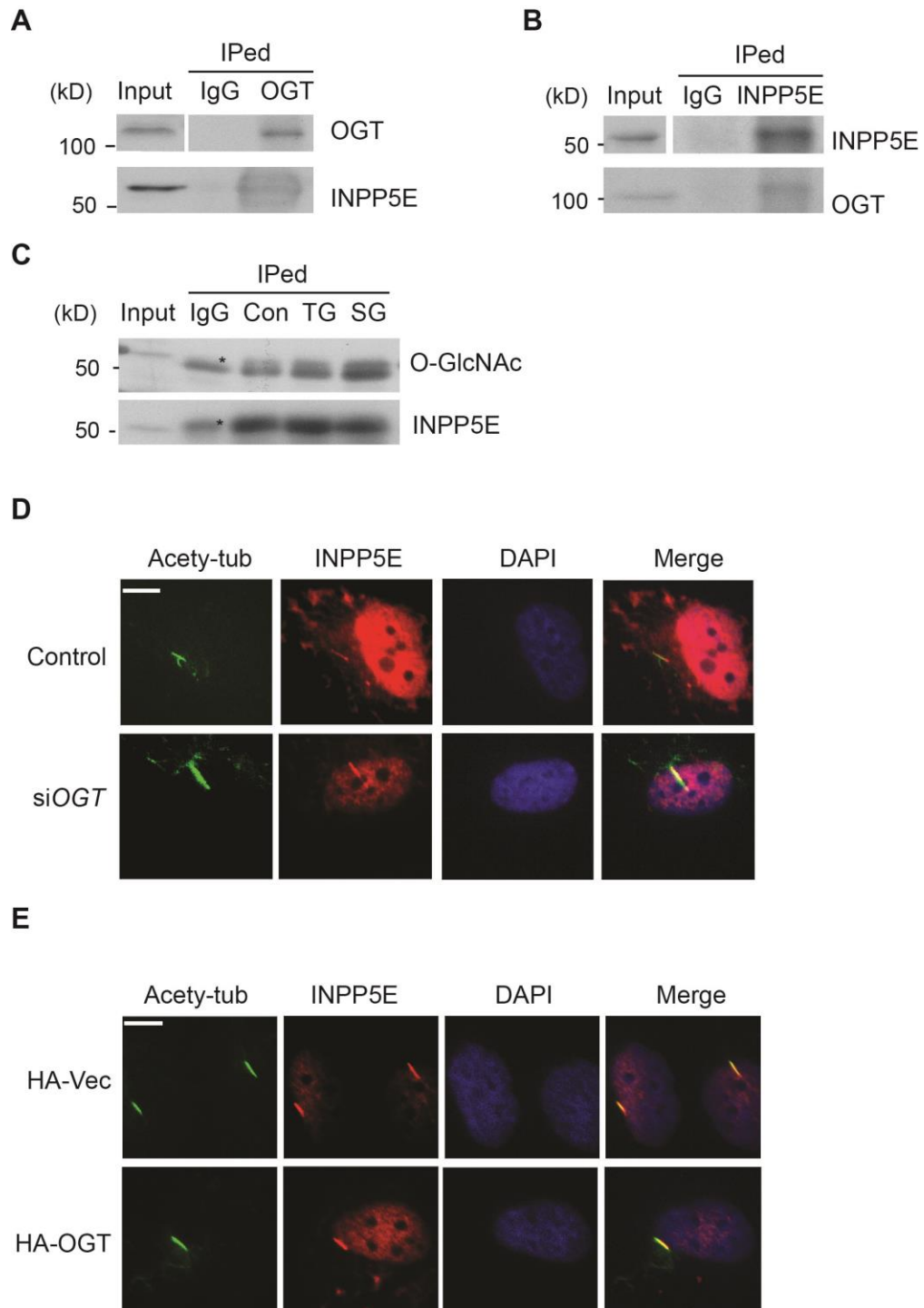## O-GlcNAcylation Regulates Primary Ciliary

Length by Promoting Microtubule Disassembly

Jie L. Tian and Hongmin Qin

Supplemental Figure

Figure S1

A

B

C

D

E

Figure S1. O-GlcNAcylation of INPP5E does not affect ciliary localization in hTERT-RPE1 cells.

(A) Endogenous OGT was IPed and subjected to IB with OGT and INPP5E antibodies.

(B) Endogenous INPP5E was IPed and subjected to IB with INPP5E and OGT antibodies.

(C) INPP5E was O-GlcNAcylated. The hTERT-RPE1 cells were first treated with mock DMSO (Con), or OGA inhibitors TG or SG, to modulate intracellular O-GlcNAc levels. Then the endogenous INPP5E was IPed and subjected to IB with O-GlcNAc and INPP5E antibodies.

(D) Representative confocal images of hTERT-RPE1 cells (si*OGT* treatment) immunostained with anti-INPP5E and anti-acetylated-tubulin antibodies. Scale bar equals 5 μm.

(E) Representative images of cells immunostained with anti-INPP5E and anti-acetylated-tubulin. The hTERT-RPE1 cells were transfected with HA-Vector or HA-OGT. Scale bar equals 5 μm.

Table S1. Antibodies used in this study

Antibody	Dilution		Source
	IB	IF	
O-GlcNAc (RL2)	1:1000	NA	Santa Cruz sc-59624
OGT (H-300)	1:1000	NA	Santa Cruz sc-32921
HDAC6 (D2E5)	1:1000	NA	Cell Signaling 7558S
IFT88	1:1000	1:200	Proteintech 13967-I-AP
Acetylated-tubulin	1:5000	1:1000	Sigma-Aldrich 017M-4806
α-Tubulin (B-7)	1:100	NA	Santa Cruz sc-5286
HA (3F10)	1:200	1:100	Sigma-Aldrich 12013819001
β-actin (C4)	1:10000	NA	Santa Cruz sc-47778
INPP5E	1:1000	1:200	Proteintech 17797-I-AP

IF, immunofluorescence; NA, not application; IB, Immunoblotting.

Transparent Methods

Cell culture

The mammalian hTERT-RPE1 (ATCC CRL-4000) and IMCD3 (ATCC CRL-2123) cells were grown in DMEM-F12 (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C in a 5% CO_2 incubator. Cilia formation was induced by serum starvation for 24 h when cells reached 80% confluency.

The *Chlamydomonas* wild type cc125 and *fla10^{ts}* strains were obtained from the *Chlamydomonas* Center (http://chlamycollection.org/). Strains were cultured and maintained on Tris-acetate-phosphate (TAP) plates. Unless otherwise specified, liquid cultures used TAP media with constant aeration in a Conviron environmental chamber at 21°C with continuous light.

Transfection

Mammalian expression vectors pcDNA3.0-HA-OGT and pcDNA3.0-HA-OGT-rescue plasmids (Li et al., 2017) were used for transfections. For IP experiments, the hTERT-RPE1 cells were first grew to 50-60% confluency. For each 10 cm Petri dish, cells were transfected with 5 μg plasmids using the TurboFect transfection reagent (Thermo Scientific) and then cultured for 48h before collected and prepared for immunoprecipitation (IP) using antibodies as indicated in the results part. For IF experiments, the hTERT-RPE1 cells were transfected with 1 μg plasmids for each 3.5 cm Petri dish as described above. The IMCD3 cells were transfected with 1 μg plasmids with Polyethylenimine (PEI) (Polysciences) reagent (Boussif et al., 1995) and then cultured for 48 h before used for IF analysis.

RNA interference and inhibitor treatment

The small RNA interference experiments were carried out using the same method as described in a prior publication (Elbashir et al., 2001). The transfection of the hTERT-RPE1 cells was carried out by using the Lipofectamine^{TM} RNAiMax Reagent (Invitrogen) according to the manufacture's protocol. A mixture of two OGT oligonucleotides (si*OGT* 1# and si*OGT* 3# (Li et al., 2017)) at 1:1 ratio was used. All oligonucleotides including the control siGl2 (5'-CGUACGCGGAAUACUUCGAdTdT-3') (Tian et al., 2016) and the si*OGT* duplexes were synthesized by Dharmacon.

The concentrations and durations of chemical inhibitor treatments used in all experiments were: TG (5 μM, 24 h), SG (1 μM, 24 h), Alloxan (5 mM, 18 h), and Tubacin (2 μM, 24 h). The solvent DMSO was used as control for each inhibitor treatments.

Rescue with HA-OGT-rescue

The hTERT-RPE1 cells were first transfected with the HA-OGT-rescue plasmid, or a control construct expressing the HA tag only (Li et al., 2017), and then cultured for 24 h followed by a 12 h treatment of siGl2 or si*OGT*. After starving in DMEM for 24 h, the cells were harvested for immunoblotting (IB) or IF analysis (Fig. 1B).

Immunoprecipitation (IP)

For IP, cells were lysed with IP buffer (50 mM Tris·Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% NP40) supplemented with a Protease Inhibitor Cocktail (Roche). Lysates were incubated with protein A/G sepharose beads (Santa Cruz) for 2 h for pre-clearing of non-specific bindings prior to incubation with primary antibodies overnight at 4°C. The beads in the pellets were then subjected to IB analysis.

Antibodies and immunoblotting (IB) assay

Table S1 contains information of primary antibodies used in this study. The methods for SDS-PAGE and IB assays were the same as previous described (Tian et al., 2016). Chemiluminescence was used to detect the primary antibodies.

Immunofluorescence staining

Cells grown on glass coverslips were fixed with 4% paraformaldehyde for 10 min, followed by fixation in ice-cold methanol for 3 min. The cells were permeabilized with 1% Triton X-100 in PBS for 10 min before blocked in PBS with 3% BSA (Pugacheva et al., 2007). Primary antibodies are listed in Table S1. Secondary antibodies of Alexa-488 and Alexa-561 were purchased from Invitrogen. Images were captured with an Olympus IX81 microscope (Olympus, Tokyo, Japan) with a Yokogawa CSU-X1 Spining Disk Unit (Andor Technology, CT, USA).

Flagella isolation and axoneme preparation

Flagella were isolated as previously described (Cole et al., 1988). Flagellar membrane was removed by incubating isolated flagella in 1% NP40 for 30 min on ice.

Tubulin deacetylase (TDAC) assay

Endogenous HDAC6 proteins were IPed from lysates of hTERT-RPE1 cells treated with TG, SG, or control DMSO. The IPs by rabbit IgG and anti-HDAC6 (Con) from control treated cell lysates were used as negative controls. TDAC assays were carried out as previously described (Hubbert et al., 2002). Briefly, *Chlamydomonas* flagella axonemes (~ 40 μg) and HDAC6 were incubated together in 200 μl TDAC buffer (20 mM Tris-Cl pH 8.0, 20 mM NaCl) at 37°C for 2 h, and then transferred to ice for 15 mins. Pellets containing protein-A beads were collected by centrifugation. The pellets were analyzed by IB with anti-HDAC6 antibody and the supernatants were analyzed with anti-α-tubulin and anti-acetylated-tubulin antibodies.

In vitro tubulin O-GlcNAcylation assay

The *in vitro* tubulin O-GlcNAcylation assays were performed as described previously (Kreppel and Hart, 1999) with minor modifications. HA-OGT expressed in hTERT-RPE1 cells was purified by IP using anti-HA antibody. The reaction mixture for *in vitro* glycosylation contained 50mM Tris·Cl, pH 8.0, 5 mM MgCl2, 2 μg HA-OGT, 6 μl flagella axonemes, and 1 mM of UDP-GlcNAc (Sigma-Aldrich) in a reaction volume of 50 μl. The reactions were performed at 25°C for 1 h or 2 h. The axonemes and beads were collected by centrifugation. Both the pellets and the supernatants were analyzed by IB with antibodies indicated in results.

Percentages of ciliated cells and ciliary length measurements

For counting the percentages of ciliated cells, only the cells clearly IF stained by antibodies against aceylated-tubulin and IFT88 were considered as ciliated. No less than 150 random selected cells combined from three independent experiments were used. The IF staining of IFT88 was used to measure the ciliary length since the staining clearly marked the base and the tip of a cilium. The segmented line selection tool of Image J (Java 1.8.0_172, https://imagej.nih.gov/ij) was used for length measurements. For all ciliary length measurements, 150 cilia combined from three independent experiments were used for statistical analysis.

Statistical analysis

Statistical analyses were performed using the software GraphPad Prism 7.0e (GraphPad software, San Diego, California, USA). Results were shown as mean ± standard deviation. Statistical analyses between two groups were performed using the student's *t*-test. p-value less

than 0.5 was considered significant. The mark "*" represents p<*0.05*, **represents p<*0.01*, and "ns" represents no significant difference.