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
O-GlcNAcylation Regulates Primary Ciliary Length by Promoting Microtubule Disassembly

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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; Suzu 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 (Suzu 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).
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 activates 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 siOGT (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 siOGT, 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 siOGT 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-GlcNAcylated positively affected ciliary assembly, promoting both cilia formation and elongation.

To verify that the effects seen in siOGT-treated cells were indeed caused by the reduction of protein O-GlcNAcylation, an siOGT-resistant OGT (HA-OGT-rescue) (Li et al., 2017) was expressed in siOGT 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 siOGT, but not in cells transfected with siGL2. The siOGT-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 siOGT knockdown cells shortened the ciliary length or not (Figure 1, B2 and 1B3). Indeed, expression of HA-OGT in OGT-depleted siOGT-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
shorter than that of the siOGT cells (4.99 ± 0.31 μm). These results confirmed that protein O-GlcNAcylation catalyzed by OGT was responsible for the elongated cilia seen in siOGT 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 siOGT (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
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.
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 ± 0.11 μm 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,
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 (labeled as O-GlcNAc). After 1 or 2 h, the supernatants and pellets were separated for IB analysis. (C) Flagella of fla10Δ 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 fla10Δ flagella from cells incubated at 32°C. The disappearance of IFT46 was used to verify that flagella were undergoing disassembly.
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 pro-
moted disassociation of tubulin from axonemal microtubules.

We reasoned that because O-GlcNAcylation promoted axonemal microtubule disassembly, cilia undergo-
ing disassembly were likely to have a higher level of O-GlcNAcylation. To test this prediction, we turned to a
temperature-sensitive flagellar mutant fla10ts of the green algae *Chlamydomonas reinhardtii*. The mutant
fla10ts 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 disas-
sembly (Cole et al., 1998; Walther et al., 1994). We checked the levels of protein O-GlcNAcylation in
fla10ts 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-GlcNAcylated protein (Gurel et al., 2014). It is possible that O-GlcNAcylation activates HDAC6 deace-
tylase 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 sub-
strate 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 pro-
tein was higher when O-GlcNAcylation was promoted by OGT overexpression (Figure 6C) or by OGA in-
hibition (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 IN-
PP5E 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 ap-
ppeared to be normal in either OGT loss- or gain-of-function cells (Figures S1D and S1E).
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 dissociation 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
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
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; Wioga 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-GlcNAcylation 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.

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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 Information

Supplemental Figure

Figure S1

A

| (kD) | Input | IgG | OGT | IPed |
|------|-------|-----|-----|------|
| 100  |       |     |     |      |
| 50   |       |     |     |      |
|      |       |     |     |      |

B

| (kD) | Input | IgG | INPP5E | IPed |
|------|-------|-----|--------|------|
| 50   |       |     |        |      |
| 100  |       |     |        |      |
|      |       |     |        |      |

C

| (kD) | Input | IgG | Con | TG | SG | IPed |
|------|-------|-----|-----|----|----|------|
| 50   |       |     |     |    |    |      |
| 50   |       |     |     |    |    |      |

D

Control

| Acety-tub | INPP5E | DAPI | Merge |
|-----------|--------|------|-------|

siOGT

| Acety-tub | INPP5E | DAPI | Merge |
|-----------|--------|------|-------|

E

| Acety-tub | INPP5E | DAPI | Merge |
|-----------|--------|------|-------|

HA-Veg

| Acety-tub | INPP5E | DAPI | Merge |
|-----------|--------|------|-------|

HA-OGT

| Acety-tub | INPP5E | DAPI | Merge |
|-----------|--------|------|-------|
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 (siOGT 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% CO₂ incubator. Cilia formation was induced by serum starvation for 24 h when cells reached 80% confluency.

The *Chlamydomonas* wild type cc125 and *fla10*<sup>ts</sup> strains were obtained from the *Chlamydomonas* Center ([http://chlamycollection.org/](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](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC5630199/)) 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](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC6743675/)) 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](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3584476/)). The transfection of the hTERT-RPE1 cells was carried out by using the Lipofectamine™ RNAiMax Reagent (Invitrogen) according to the manufacture’s protocol. A mixture of two OGT oligonucleotides (siOGT 1# and siOGT 3# ([Li et al., 2017](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC5630199/))) at 1:1 ratio was used. All oligonucleotides including the control siGI2 (5'-CGUACGCGGAUUACUCGAdTdT-3') ([Tian et al., 2016](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4951674/)) and the siOGT duplexes were synthesized by Dharmaco.
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 siOGT. 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 acetylated-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.