A genetic model to study O-GlcNAc cycling in immortalized mouse embryonic fibroblasts

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O-GlcNAcycling is an abundant posttranslational protein modification in which the monosaccharide O-GlcNAc is added to Ser/Thr residues by O-GlcNAc transferase and removed by O-GlcNAcase. Analyses of O-GlcNAc-mediated signaling and metabolic phenomena are complicated by factors including unsatisfactory inhibitors and loss-of-function cell lines lacking identical genetic backgrounds. In this work, we generated immortalized WT, Oga knockout, and Ogt floxed allele (Ogt floxed) mouse embryonic fibroblast (MEF) cell lines with similar genetic backgrounds. These lines will facilitate experiments and serve as a platform to study O-GlcNAc cycling in mammals. As a test paradigm, we used the immortalized MEF lines to investigate how changes in O-GlcNAcycling affect the phosphorylation of the tau protein. The activity of glycogen synthase kinase 3β (GSK3β), a kinase that phosphorylates tau, decreases when expressed in Oga knockout MEFs compared with WT cells. Phosphorylation at Thr231 in recombinant, tauopathy-associated tau with a proline-to-leucine mutation at position 301 (P301L) was altered when expressed in MEFs with altered O-GlcNAc cycling. In aggregate, our data support that O-GlcNAc cycling indirectly affects tau phosphorylation at Thr231, but tau phosphorylation was highly variable, even in genetically stable, immortalized MEF cells. The variable nature of tau phosphorylation observed here supports the need to use cells akin to those generated here with genetically defined lesions and similar backgrounds to study complex biological processes.

Addition of the monosaccharide β-N-acetyl-α-glucosamine to nuclear and cytoplasmic Ser/Thr residues by O-GlcNAc transferase (OGT)2 yields the posttranslational modification O-GlcNAc, which can be removed by O-GlcNAcase (OGA). Thousands of proteins are modified with O-GlcNAc, and O-GlcNAcycling is thought to play a role in regulating many major cellular processes, such as cell cycle progression (1–3), transcriptional control (4–7), signal transduction (8, 9), nutrient sensing (10–12), stress response (13, 14), and chromatin remodeling (7, 15–17). The involvement of O-GlcNAcycling in diverse and interdependent pathways makes the biology of O-GlcNAcycling extremely complex (18). To simplify the analysis, we generated immortalized mouse embryonic fibroblasts (MEFs) with unique genotypes and a shared genetic background to serve as a genetically stable platform to study O-GlcNAc cycling in mammals. Beyond the study of cellular processes, these MEFs may be used for recombinant expression of proteins akin to the well characterized, lectin-resistant Chinese hamster ovary lines widely used for recombinant expression of biopharmaceuticals.

Standard genetic models for O-GlcNAcycling have been difficult to generate in mammals, as loss of OGT is lethal at the single-cell level, and Oga knockout (KO) results in low viability of embryos or embryonic lethality. Currently, researchers use chemical inhibitors (19, 20–23) and siRNA (24, 25) to study aberrant O-GlcNAcycling in mammals. Although these methods continue to prove useful, inhibitors have the potential for off-target effects, and complete siRNA knockdown plagues the field. The cell lines described here, with genetic deletion of Ogt or Oga, could greatly simplify the analysis of O-GlcNAcycling.

Oga KO (26) and Ogt floxed allele (26) mutants have been generated in mice, as have primary MEFs derived from these lines. However, routine generation of primary MEFs is time-consuming (2–3 weeks to generate) and introduces considerable bias because of differences between genetic backgrounds and passage-dependent changes in the primary line. When generated, the primary MEFs reach their Hayflick limit after only five to seven passages, further limiting the window during which primary MEFs can be used for experiments. Conditional Ogt KO immortalized cells with inducible Cre (iCre) recombinase expression have been generated (27), simplifying analysis with tightly controlled Cre recombinase expression. Here we describe the first paired MEF lines available for exploring loss of O-GlcNAcase. However, differences in the genetic backgrounds of previously described MEF lines may not allow robust comparison and analysis.

Considering the limitations of the current reagents available, we generated immortalized WT, Oga KO, and Ogt floxed allele...
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MEF cell lines on a common C57B6 background that can be used as a platform to study O-GlcNAc cycling in mammals. As genetic background can lead to tremendous variability in phenotype (28), the three genotypes described here offer the advantage of a shared genetic background, which is lacking in previously generated MEFs (27). The immortalized MEF lines can be grown for as many as 50 passages, with comparable biological behavior at each passage. The ease of culture maintenance as well as the generation of multiple genetic siblings for each genotype support increased replicates with defined genetic backgrounds, thereby enabling assessment of biologically relevant variation. Of note, in the Ogt floxed MEF line, Ogt is flanked on either end by a lox P site, and addition of Cre recombinase lines catalyzes the excision of the Ogt. In addition to the cell line requiring an external source of Cre recombinase (AdCre), we created an Ogt floxed allele MEF line with an inducible Cre recombinase (iCre).

After generating these cell lines, we sought to demonstrate the utility of the immortalized MEFs with WT, Oga KO, and Ogt floxed allele genotypes. Posttranslational modifications of tau, such as O-GlcNAcylation and phosphorylation, are thought to influence neurodegeneration-associated tau aggregation and neurofibril tangles (29–31). As explored by others, we evaluated tau posttranslational modifications and the activity of glycogen synthase kinase 3β (GSK3β), a kinase known to act on tau. Tau phosphorylation is simply one example to demonstrate that the ease of culture maintenance, comparable biological behavior even after extended passaging, and genetic sibling background make the immortalized MEF cell lines described here an excellent system to study the role of O-GlcNAc cycling in mammalian biological processes.

**Results**

**Generation of immortalized MEFs as a platform to study O-GlcNAc cycling**

MEFs were immortalized via lentiviral SV40 large T antigen infection—Ogt floxed and Oga KO mouse strains were extensively backcrossed with C57B6 for a least five generations to attain a consistent genetic background differing only at the genetically altered region. Primary MEFs were isolated from each strain at embryonic day 13.5, and sibling pairs of the associated genotypes were identified. Primary MEFs with WT, Ogt floxed, and Oga KO genotypes were immortalized via lentiviral infection with SV40 large T antigen. To enable the study of X-linked properties associated with Ogt, male and female lines were generated for each genotype. In addition, primary MEFs generated from two to four different embryos were immortalized for each genotype to facilitate the study of biological variability associated with O-GlcNAc cycling. The generated conditional Ogt KO and genetic siblings offer useful biological variant information that cannot be attained with the genetically identical variants currently generated with CRISPER/Cas.

Fig. 1, a–c, shows the viable cell count and percent viability for a representative immortalized MEF line for each genotype over 200 days. Each cell line underwent ~50 passages during the 200-day observation period. Each immortalized line shows stable and robust growth with an average doubling rate of 25 h for more than 200 days with viability greater than 90%. In addition to observing cell viability across multiple passages, we also looked at the viability of the immortalized MEFs within a single passage. Fetal bovine serum (FBS) interferes with adenoviral Cre (AdCre) recombinase transduction. Thus, we monitored immortalized MEFs using medium with FBS (i.e. DMEM) versus medium without FBS (i.e. Opti-MEM). Fig. 1d shows that WT and Oga KO immortalized MEFs retain 60%+ viability over 7 days of culturing in Opti-MEM and DMEM. Ogt floxed lines grown in Opti-MEM maintain roughly 40% viability over time with and without addition of AdCre recombinase (Fig. 1e). Given the lower viability of the Ogt floxed lines, Ogt floxed cells with (+) or without (−) adenoviral Cre recombinase were stained with propidium iodide (PI) and Annexin V (AV) to identify the percentage of necrotic and apoptotic cells over the course of a passage. The percentage of healthy cells was low, remaining at 10% over 7 culture days. Despite this, the cells were still useful and were effectively transfected with recombinant proteins such as GSK3β and tau as described later.

**Ogt knockout generated with AdCre recombinase**—Knockout of OGT is lethal in mammalian systems at the level of the individual cell. For the experiments outlined here, Ogt KO in Ogt floxed MEFs was induced with infection via AdCre recombinase. When expressed, Cre recombinase excises the Ogt gene. OGT levels were monitored in Ogt floxed MEFs for 8 days following AdCre infection (Fig. 2a). The maximum reduction in OGT was observed on day 8 after AdCre infection, with a decrease of 91% relative to the day 4 control. Experiments were replicated in four Ogt floxed MEF genetic sibling lines to assess the effect of biological variability and gender on the range of Ogt KO achieved following AdCre infection. Loss of OGT across the two biological replicates of four genetic sibling Ogt floxed MEF lines is depicted in Table 1. O-GlcNAc levels in Ogt floxed MEFs were reduced by 50% on day 4 following AdCre infection (Fig. 2b). Even though OGT and O-GlcNAc levels were reduced following AdCre infection, cell viability was low by day 4 (Fig. 1, e and f, and Table 1). Despite a series of experiments to optimize experimental conditions for Ogt knockdown, viability remained low, as would be expected given that OGT is essential for cellular metabolism.

**CreER line for tamoxifen-dependent inducible Ogt knock-out**—Although AdCre-recombinase effectively excised Ogt in the immortalized Ogt floxed MEFs, culturing conditions were not ideal given the high percentage of apoptotic and necrotic cells found by the time the OGT KO was achieved. Kazemi et al. (27) created MEFs with an iCre plasmid so that Ogt excision can be induced using hydroxytamoxifen treatment. Here we demonstrate that a similar approach can be used for the immortalized MEFs. We transformed the immortalized Ogt floxed MEFs discussed under “Ogt knockout generated with AdCre Recombinase” with an iCre plasmid. Following treatment with 0.5 μM hydroxytamoxifen, we observed absence of OGT by 48 h after treatment (Fig. 3). Further, although loss of OGT expression was 100%, viability at 48 h also remained...
After 72 h of treatment with 0.5 μM HT, cell viability was reduced to 75%. Therefore, we recommend Ogt floxed MEFS with iCre recombinase within 48 h following 0.5 μM HT treatment to ensure maximal OGT knockdown with high cell viability.

Oga knockout—Oga (mgea5) KO was confirmed for all immortalized MEF lines by genotyping and an enzyme activity assay. Negligible OGA activity was observed in immortalized Oga KO MEFS (32, 33), and loss of OGA was observed by Western blotting, as was an increase in O-GlcNAc levels compared with the WT (Fig. 4).

Figure 1. a–c, viable cell counts and percent viability of representative immortalized WT (a), Oga KO (b), and Ogt floxed allele (c) MEF cell lines generated via lentiviral infection with SV40 large T antigen. Viable cell counts were measured directly before (splitting density) and after (plating density) passaging. Immortalized MEF lines were maintained for more than 50 passages with an average doubling rate of 25 h and viability of more than 90%. d, viability of immortalized MEFs when cultured using medium with FBS (i.e. DMEM) versus medium without FBS (i.e. Opti-MEM). WT and Oga KO cells retain 60%+ viability over 7 days of culturing in Opti-MEM and DMEM. e, Ogt floxed lines grown in Opti-MEM maintain roughly 40% viability over time with (+) or without (−) addition of AdCre. f, Ogt floxed cells with (+) or without (−) AdCre stained with PI and AV to identify the percentage of necrotic and apoptotic cells over 7 culture days.

Figure 2. a, Western blot of Ogt floxed MEFs over time after infection with AdCre (+). % Δ OGT indicates the percentage of OGT reduction calculated from the average of 2 culture day 4 (D4) samples without AdCre (−). b, Western blotting of female and male Ogt floxed MEFs with and without addition of AdCre, demonstrating O-GlcNAc and OGT levels over time. By day 4 after infection with adenoviral Cre recombinase, O-GlcNAc levels were reduced by roughly 50%. Day 0 is the day when AdCre was added, day 1 was 24 h after addition, etc.
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Table 1

| Cell line          | Gender | Post-Cre | %V | %ΔOGT |
|--------------------|--------|----------|----|-------|
| Ogt floxed 1       | ?      | D0       | 97 | –     |
| Ogt floxed 4       | ♂      | D0       | 95 | –     |
| Ogt floxed 1       | ?      | D1       | 90 | –18   |
| Ogt floxed 4       | ♂      | D1       | 97 | –12   |
| Ogt floxed 1       | ?      | D4       | 55 | –86   |
| Ogt floxed 4       | ♂      | D4       | 30 | –85   |
| Ogt floxed 1       | ?      | D7       | 31 | –83   |
| Ogt floxed 4       | ♂      | D7       | 23 | –65   |
| Ogt floxed 1       | ?      | D8       | 37 ± 29 | –95 ± 5 |
| Ogt floxed 3       | ♂      | D8       | 12 | –100  |
| Ogt floxed 4       | ♂      | D8       | 23 ± 5 | –99 ± 1 |
| Ogt floxed 5       | ♂      | D8       | 31 ± 17 | –100 ± 0 |

**Figure 3.** a, Western blot analysis of Ogt floxed MEFs with iCre after 48 and 72 h of treatment with 0.5 μM HT. b, cell viability (%V) and percentage of OGT knockdown (%ΔOGT) for the blot shown in a. 100% OGT knockdown was observed after 48 h of treatment with 0.5 μM HT. Cell viability remained >90% at 48 h. After 72 h of treatment with 0.5 μM HT, although OGT knockdown was substantial, cell viability was 75%. Data shown are for one biological replicate.

**Utility of immortalized MEFs**

O-GlcNAc decreases the activity of recombinant GSK3β—To demonstrate the utility of the immortalized MEF lines, we investigated the impact of O-GlcNAc cycling on the activity of a known substrate of OGT, GSK3β. The kinase GSK3β is fundamental to development and insulin signaling and is known to phosphorylate tau. In our previous work (32), we observed that primary Oga KO MEFs showed increased Ser9 phosphorylation of GSK3β compared with the WT. We found the same increased GSK3β Ser9 phosphorylation with recombinantly expressed, epitope-tagged GSK3β in our Oga KO, SV40-transduced MEFs (Fig. 5a). Although Oga KO increased GSK3β phosphorylation at Ser9 compared with the WT, loss of OGT in Ogt floxed MEFs + AdCre did not yield statistically significant changes from the control because of the wide variation observed (Fig. 5b). Given these findings, along with the data from Fig. 1d, we focused on Oga KO MEFs in subsequent experiments with GSK3β.

Next, we monitored the HA-GSK3β kinase activity of the recombinant enzyme expressed in WT and Oga KO MEFs. The activity of affinity-purified HA-GSK3β was monitored (the rate of ATP to ADP conversion) via a luminescent assay. We first assessed the effect of initial ATP concentration on the rate of ATP to ADP conversion when the concentration of purified recombinant GSK3β purified from Oga KO cells and WT cells was held constant at 24 ng/μl (Fig. 6a). At nearly saturating concentrations of ATP, both enzymes exhibited the same rates of ATP conversion (Fig. 6a). The K_m values with respect to ATP were nearly identical between the two enzymes (Oga KO–derived K_m = 38). We then varied the concentration of recombinant kinase when initial ATP concentration was held constant at 1 μM ATP and observed a linear increase in the rate of ATP-to-ADP conversion with increasing kinase concentrations for both Oga KO and WT MEFs (Fig. 6b). The specific activity of the Oga KO GSK3β was decreased by ~2-fold over a range of enzyme concentrations from 4–24 ng/μl. The decrease in ATP conversion observed for GSK3β produced in Oga KO MEFs compared with the WT suggests that loss of O-GlcNAc cycling is associated with both increased phosphorylation of GSK3β at Ser-9 (Fig. 5a) but also decreased specific activity of GSK3β, consistent with enhanced Ser9 phosphorylation.

O-GlcNAc cycling and tau phosphorylation—Hypophosphorylation of tau has been linked to its role in neurodegeneration. For example, Thr231 of GSK3β has been shown to play a critical role in regulating tau’s ability to bind and stabilize microtubules (34). Given that changes in O-GlcNAc cycling affect the activity of GSK3β, a kinase known to modify tau, we wanted to explore whether tau phosphorylation is altered when OGT or OGA expression is altered. Indeed, others have demonstrated, in multiple mouse and human cell lines, that tau phosphorylation is modulated by changes in O-GlcNAcylation (30, 35–37); however, these studies have assumed a genetically homogenous background.

Phosphorylation of tau at Thr231, Ser396, Ser262, and AT8 (Ser202 and Thr205) was examined via expressing recombinant...
tauopathy-associated tau(P301L) in four Ogt floxed immortalized MEF lines from genetic siblings. We also performed the transfections in two WT and Oga KO immortalized MEF lines who were littermates. Experiments were performed at least twice for each cell line to assess the variability within the cell line and between the cell lines. When O-GlcNAc cycling was disrupted and tau pThr231 was examined, loss of both OGT and OGA showed a change in phosphorylation that trended downward (Fig. 7). These findings are consistent with the change in GSK3β activity upon loss of Oga (Fig. 6). Interestingly, we demonstrate that loss of OGA also corresponds with a decrease in OGT under these culturing conditions, which demonstrates the complexity of understanding a change in O-GlcNAc cycling even in a genetically defined background.

Despite use of a genetically stable and reproducible system, we observed no clear reproducible change in phosphorylation for the Ogt floxed MEFs treated with and without AdCre or Oga KO versus WT for Ser196, Ser262, and AT8 (Fig. S1). Given the large degree of intrinsic variability we observe even between MEF line biological replicates having the same genetic background, we suggest that additional experimental optimization may be required to tease out the role of O-GlcNAc cycling in tau phosphorylation when performing this kind of experiment in an Ogt loss-of-function background.

Figure 5. a and b: phosphorylation of Ser9 on recombinant GSK3β was increased in Oga KO MEFs relative to the WT (a), but no statistically significant difference was seen for Ogt floxed MEFs treated with and without AdCre (b). Each lane represents a unique cell line, with three sets of genetic siblings shown for each genotype. Data shown in the bar chart are normalized pSer9 divided by normalized rGSK3β (n = 3, error bars = range). In b, the molecular marker 15 kDa is at the bottom of the gel because the second band of H3 ran off the gel.

Figure 6. a, ATP converted by GSK3β purified using the epitope tag from WT and Oga KO MEFs in the presence of 24 ng/µl GSK3β. GSK3β ATP conversion is similar for the enzyme purified from WT MEFs and Oga KO MEFs. Error bars represent standard deviations derived from averages of triplicate determinations at each concentration. b, ATP converted in WT and Oga KO MEFs using various concentrations of GSK3β with initial concentration of ATP at 1 µM. Data are derived from three biological replicates. Error bars indicate the standard error of the mean for the three determinations.
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Figure 7. a, cartoon showing the location of various phosphorylation sites on tau protein (AT8, pThr231, pSer262, pSer396, and pSer422). b and c, tau pThr231 was detected in immortalized WT, Oga KO, and Ogt floxed MEFs (with or without AdCre). d, graphed data represent between two to four biological replicates for each MEF line (indicated by numbers for Ogt floxed lines). Phosphorylation at Thr231 is decreased for MEFs in which O-GlcNAc cycling is compromised. Each point represents the average of the technical replicates in b and c, with blue and pink points representing male and female samples, respectively. Error bars represent mean and standard deviation (d). Despite the genetically stable system, inherent variance across biological replicates remains. In c, the molecular marker 15 kDa is at the bottom of the gel because the second band of H3 ran off the gel.

Discussion

The immortalized MEFs generated in this work were envisioned to provide a genetically stable platform to study O-GlcNAc cycling, with potential applications including verification of antibodies directed against OGT and OGA, investigation of the specificity of OGT and OGA inhibitors, and recombinant protein production with defined O-GlcNAcylation status. Indeed, we have demonstrated the specificity of multiple antibodies (listed under “Materials and methods”) using the Ogt floxed, WT, and Oga KO cell lines. Importantly, these loss-of-function lines are routinely used in the laboratory to confirm lot-to-lot variability of antibodies acquired. Further, as glycosylation of recombinant proteins has proven useful with lectin-resistant Chinese hamster ovary cells, we demonstrated that recombinantly expressed GSK3β could be purified and its activity monitored under different glycosylation states.

We have also shown the utility of Ogt floxed, WT, and Oga KO MEFs by probing GSK3β activity and phosphorylation of tau P301L, a tauopathy-associated tau with proline-to-leucine mutation at position 301. Our data support a complex, potentially competitive relationship between O-GlcNAcylation and phosphorylation (30, 36, 37). Unfortunately, the impact of O-GlcNAc cycling on tau phosphorylation was highly variable. The variable nature of tau phosphorylation with a genetically stable system suggests that care must be taken when interpreting the phosphorylation of tau, as results may be difficult to reproduce, even with a genetically stable model system. Further, optimization of experiments using cell lines in which loss of Ogt can be induced by compounds such as HT will clearly prove fruitful. Intriguingly, we find that, even with genetically defined MEFs on a constant genetic background with targeted alleles of Oga and Ogt, compensation in the variable nature of tau phosphorylation with a genetically stable system suggests that care must be taken when interpreting the phosphorylation of tau, as results may be difficult to reproduce, even with a genetically stable model system. Further, optimization of experiments using cell lines in which loss of Ogt can be induced by compounds such as HT will clearly prove fruitful. Intriguingly, we find that, even with genetically defined MEFs on a constant genetic background with targeted alleles of Oga and Ogt, compensation in the levels of the two relevant enzymes alters O-GlcNAcylation. However, generation of such cell lines is important to understand the mechanism of such compensation and to move toward a more uniform way to produce biologic agents with altered O-GlcNAcylation.

Experimental procedures

MEF isolation

MEF isolation was performed according to the method described by Xu (39). Briefly, mouse breeder pairs were set up, and females were checked for copulatory plugs the following morning. Plugged females were removed from male breeder cages. On embryonic day 13.5, the pregnant female mouse was anesthetized with CO₂ gas and euthanized via cervical dislocation, and the intact uterus was extracted and cut into sections between each embryo. Each embryo was separated from the placenta, membranes, and umbilical cord. The bulk of the central nervous system tissue was removed by severing the head above the level of the oral cavity and saved for genotyping. Forsps were used to remove the dark red tissue so that the majority of remaining cells were fibroblasts. In a new Petri dish, the embryo body was minced in the presence of trypsin. The minced tissue was transferred to a 15-ml conical tube with 5 ml of culture medium and centrifuged. The supernatant was aspirated, and the tissue was washed with 1× PBS. The pellet was resuspended in 2 ml of MEF culture medium (DMEM with 10% FBS and 1% penicillin/streptomycin). The embryonic cell and medium mixture was then transferred to tissue culture dishes with fresh culture medium and placed in a 37 °C incubator to grow.

MEF immortalization via SV40 infection

Primary MEFs between passages 3 and 4 were split into 6-well dishes with 2 ml of MEF culture medium and grown overnight in a 37 °C incubator with 5% CO₂ and 3% O₂. When the MEFs reached ~50% confluence, cells were considered ready for transduction. One hour before transduction, the MEF culture medium was changed. The recombinant lentiviral vec-
tor SV40 supernatant (Capitol Biosciences, CIP-0011) was thawed on ice, and MEF culture medium was aspirated. 500 μl of the viral supernatant was added to each well with 10 μg/ml Polybrene and incubated overnight at 37 °C. The viral supernatant was removed after 24 h, replaced with 2 ml of MEF culture medium, and incubated at 37 °C until just confluent, ~48–72 h after transduction. When confluent, the MEFs were subcultured into a 10-cm tissue culture dish with 10 ml of MEF culture medium; this was considered passage 1. The MEFs were split every 3–4 days for 10–15 days after passage 1, and then SV40-transformed clones were selected, isolated using cloning rings, and plated for expansion. Clones were confirmed for SV40 transformation via immunochemistry with the following primary and secondary antibodies: anti-SV40 T antigen antibody (Abcam, PAb416, ab16879) and donkey anti-mouse IgG H&L (Alexa Fluor® 488) (Abcam, ab150105).

Ogt floxed MEF treatment with adenoviral Cre recombinase

Immortalized Ogt floxed MEFs were plated in 6-well plates and grown overnight. When cells reached ~80% confluency, the culture medium was changed ~1 h prior to infection. Just prior to infection, MEF culture medium was aspirated and replaced with Opti-MEM (Gibco, 31985-070). Adenoviral Cre (Vector Biolabs, 1045) was added to the culture at a multiplicity of infection of 580. Nearly complete Ogt KO required incubation of at least 4 days. After incubation with AdCre, medium was collected, and adherent cells were trypsinized, combined with suspended cells from medium, pelleted at 1000 rpm, resuspended in 1× PBS, counted, repelleted, and frozen for Western blot analysis. Cell viability was assessed using trypsin blue staining of combined suspended and trypsinized cells. Live and dead cells were counted using the TC20 automated cell counter (Bio-Rad).

The percentages of necrotic and apoptotic cells were identified via staining with PI and AV according to the manufacturer’s instructions (eBioscience, 88-8007-74). In brief, cells were cultured to ultimately provide ~100,000 cells/sample. After 4 and 7 days in Opti-MEM, some cells were lifted, whereas others were adherent. All cells were combined for the experiment: lifted cells were isolated first and combined with adherent cells. Adherent cells were lifted with trypsin (uniform number of minutes to minimize changes to the cell surface with trypsin treatment), and trypsin was quenched with medium containing FBS by flowing through a filter (Gibco 542070) to remove clumped cells. Cells were resuspended/washed in 500 μl of PBS, counted by trypsin blue exclusion, and repelleted, and the supernatant was removed. Cell pellets were stored on ice until all cells were collected. For Annexin V and PI staining, cells were resuspended in binding buffer to 83,000 (total) cells/100 μl, and 100 μl was aliquoted to a V-bottomed 96-well plate. The plate was centrifuged at 2,000 rpm, and then cells were resuspended in 1× binding buffer with 5 μl of AV-APC (Annexin V-allophycocyanin) solution/100 μl and incubated at room temperature for 10 min. Cells were washed with 1× binding buffer and resuspended in 200 μl of 1× binding buffer (with 5 μl of PI/200 μl of buffer), placed on ice, and then analyzed by flow cytometry within 30 min. 10,000 cells were counted for each sample, gating for AV on separate channels. Data were analyzed in FlowJo, and the table was exported for analysis in Prism/Excel.

Generation of Ogt floxed MEFs with inducible Cre recombinase

The SV40-transformed MEFs with the Ogt floxed allele were co-transfected with plasmids pCAG-ERT2CreERT2 (Addgene, 13777) and pcDNA™3.1 (Thermo Fisher, V79020) at a 3:1 ratio. 48 h following transfection, 600 μg/ml G418 was added to the medium to select clones with neomycin resistance. It was assumed that, because of the plasmid DNA ratio, clones with the neomycin resistance from pcDNA3.1 would also be successfully transfected with pCAG-ERT2CreERT2. Single-cell clones were then isolated via cloning rings and propagated. Clones cultured in MEF culture medium were then tested via Western blotting for OGT KO following treatment with 0.5 μM hydroxytamoxifen in ethanol (HT, Sigma, H7904).

OGA activity assay

OGA activity was determined for MEF lysates as described previously by Kim et al. (38). Briefly, final concentrations of 1 mM fluorescein-di-GlcNAc and 500 mM GalNAc in 0.5 mM citrate phosphate buffer (pH 6.5) were added to 150 μg of cell lysate. Reactions were incubated in the dark at 37 °C with shaking at 100 rpm for 30 min and then quenched with 600 μl of 750 mM Na2CO3. Fluorescence was measured with a fluorometer at 485 nm excitation/535 nm emission.

Transfection with Tau P301L and GSK3β

SV40-transduced MEFs were plated in either 6-well or 10-cm tissue culture plates with 2-ml or 10-ml working volume, respectively, and grown overnight. When cells reached 50–70% confluence, AdCre was added to Ogt floxed MEFs as described above. WT and Ogt KO MEFs were grown in Opti-MEM alongside the Ogt floxed MEFs. 6 days after AdCre infection, MEFs were transfected with either the pRK5-eGFP-Tau P301L plasmid (Addgene, 46904) or the HA GSK3β WT pcDNA3 plasmid (Addgene, 14753) using Lipofectamine transfection reagent. Transfection efficiency was estimated visually by observing fluorescent cells. Consistently, more than 95% of the cells appeared fluorescent, indicating a high transfection efficiency. Following transfection, cells were incubated for 24 h at 37 °C and 3% O2. Cells were then trypsinized, counted, and pelleted. Cell pellets were lysed in T-PER Tissue Protein Extraction Reagent (Thermo Fisher, 78510) with Halt protease and phosphatase inhibitor mixture (Thermo Fisher, 78440) for further analysis.

Western blot analysis

Traditional Western blot procedures were used to determine loss of OGT and OGA and assess O-GlcNAc levels and the presence of phospho-tau epitopes and phosphorylation of GSK3β in immortalized MEFs. The following primary antibodies were used: rabbit anti-O-GlcNAc transferase (H-300, Santa Cruz Biotechnology, SC-32921), mouse anti-O-GlcNAc (Thermo Fisher, MA1-072), rabbit anti-OGA (Abcam, ab124807), rabbit anti-β-actin (Abcam, ab1801), anti-phospho-Tau (pSer206, Sigma, T 7319) anti-phospho-Tau (pSer396, Sigma, T 7944), rabbit mono- to anti-phospho-Tau (pSer214, pSer280, pSer202, pSer199).
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Bioss, bs-5416R), anti-phospho-Tau (pThr231, Sigma, T 7194), anti-phospho-Tau (pSer262, Sigma, T 7569), phospho-GSK3β (Ser9) antibody (Cell Signaling Technology, 9336s), and GSK3β antibody (Cell Signaling Technology, 9332) with the secondary antibodies Odyssey goat anti-rabbit 800CW (LI-COR, 926-32211) and Odyssey goat anti-mouse 800CW (LI-COR, 827-08364). The following loading controls were used to normalize band intensity: rabbit anti-β-actin (Bioss, bs-0061R) and rabbit anti-histone H3 (Abcam, ab1791). Blots were imaged with the Odyssey Classic IR imaging system (LI-COR, Lincoln, NE) with an excitation wavelength of 778 nm and an emission wavelength of 795 nm. Band intensities were analyzed with Image Studio software (LI-COR).

Activity of GSK3β

HA-GSK3β was purified from total lysate using the Pierce HA Tag Magnetic IP/Co-IP kit (Thermo Fisher, 88838). The activity of purified HA-GSK3β was determined using the ADP-Glo kinase assay (Promega, V6930) according to the manufacturer’s protocol. Each reaction used 1 mM GSK3 precipitated with HA-agarose beads and incubated for 40 min at room temperature. Following the kinase reaction, ADP-Glo reagent was added to the reaction mixture and incubated for 40 min at room temperature. Following the kinase reaction, ADP-Glo reagent was added to the kinase reaction mixture and incubated for 40 min at room temperature to stop the kinase reaction and deplete unconverted ATP. Then kinase detection reagent was added to convert ADP to ATP and introduce luciferase and luciferin to detect ATP. The kinase reaction was incubated with detection reagent for 60 min, and then luminescence was measured with a plate-reading luminometer. Data were analyzed using the Michaelis–Menten curve nonlinear fitting algorithm in Prism.

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