

Transcriptional regulation of O-GlcNAc homeostasis is disrupted in pancreatic cancer

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Many intracellular proteins are reversibly modified by O-linked GlcNAc (O-GlcNAc), a post-translational modification that dynamically regulates fundamental cellular processes in response to diverse environmental cues. Accumulating evidence indicates that both excess and deficiency of protein O-GlcNAcylation can have deleterious effects on the cell, suggesting that maintenance of O-GlcNAc homeostasis is essential for proper cellular function. However, the mechanisms through which O-GlcNAc homeostasis is maintained in the physiologic state and altered in the disease state have not yet been investigated. Here, we demonstrate the existence of a homeostatic mechanism involving mutual regulation of the O-GlcNAc-cycling enzymes O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) at the transcriptional level. Specifically, we found that OGA promotes *Ogt* transcription through cooperation with the histone acetyltransferase p300 and transcription factor CCAAT/enhancer-binding protein β (C/EBP β). To examine the role of mutual regulation of OGT and OGA in the disease state, we analyzed gene expression data from human cancer data sets, which revealed that *OGT* and *OGA* expression levels are highly correlated in numerous human cancers, particularly in pancreatic adenocarcinoma. Using a *Kras*^{G12D}-driven primary mouse pancreatic ductal adenocarcinoma (PDAC) cell line, we found that inhibition of extracellular signal-regulated kinase (ERK) signaling decreases OGA glycosidase activity and reduces OGT mRNA and protein levels, suggesting that ERK signaling may alter O-GlcNAc homeostasis in PDAC by modulating OGA-mediated *Ogt* transcription. Our study elucidates a transcriptional mechanism that regulates cellular O-GlcNAc homeostasis, which may lay a foundation for exploring O-GlcNAc signaling as a therapeutic target for human disease.

O-GlcNAcylation, or the attachment of single O-linked GlcNAc (O-GlcNAc)³ moieties to serine and threonine residues of cytoplasmic, nuclear, and mitochondrial proteins, is an essential post-translational modification in metazoans (1, 2). As a product of nutrient flux through the hexosamine biosynthetic pathway, protein O-GlcNAcylation couples changes in nutrient availability to dynamic regulation of fundamental cellular processes such as gene expression, metabolism, and signal transduction (1–9). Perturbations in cellular O-GlcNAc homeostasis have been linked to human disorders ranging from cancer and diabetes to cardiovascular and neurodegenerative disease, suggesting that maintenance of O-GlcNAc homeostasis is vital for normal cellular and physiological function (2, 10–14). However, what mechanisms exist to preserve cellular O-GlcNAc homeostasis under physiologic conditions is an outstanding question in the field that has remained largely unexplored.

We previously hypothesized that cellular O-GlcNAc homeostasis is maintained by mutual regulation of O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), the sole enzymes that add and remove O-GlcNAc, respectively (2). Several studies have shown that *OGT* and *OGA* mRNA levels are responsive to perturbations in O-GlcNAc signaling and that OGT and OGA are themselves O-GlcNAcylated, suggesting that mutual regulation of OGT and OGA could occur at both the transcriptional and post-translational levels (15–18). These putative homeostatic mechanisms could function to preserve the balance between OGT and OGA activity and expression and thereby maintain cellular O-GlcNAcylation levels within an “optimal zone” (2). Here, we provide evidence to support the existence of mutual regulation of OGT and OGA at the transcriptional level, demonstrating that OGA does indeed modulate *Ogt* transcription and vice versa. Furthermore, we describe a specific mechanism in which OGA, in conjunction with the histone acetyltrans-

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This article contains Tables S1–S7 and Figs. S1–S3.

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³ The abbreviations used are: O-GlcNAc, O-linked GlcNAc; OGT, O-GlcNAc transferase; OGA, O-GlcNAcase; C/EBP, CCAAT/enhancer-binding protein; PDAC, pancreatic ductal adenocarcinoma; ERK, extracellular signal-regulated kinase; HEK, human embryonic kidney; TMG, Thiamet G; ChIP-seq, ChIP sequencing; LAP, liver-enriched activator protein; LIP, liver-enriched inhibitor protein; TCGA, The Cancer Genome Atlas; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; FBS, fetal bovine serum; 4MU, 4-methylumbelliferyl; GalNAc, N-acetyl-D-galactosamine; qPCR, quantitative PCR.

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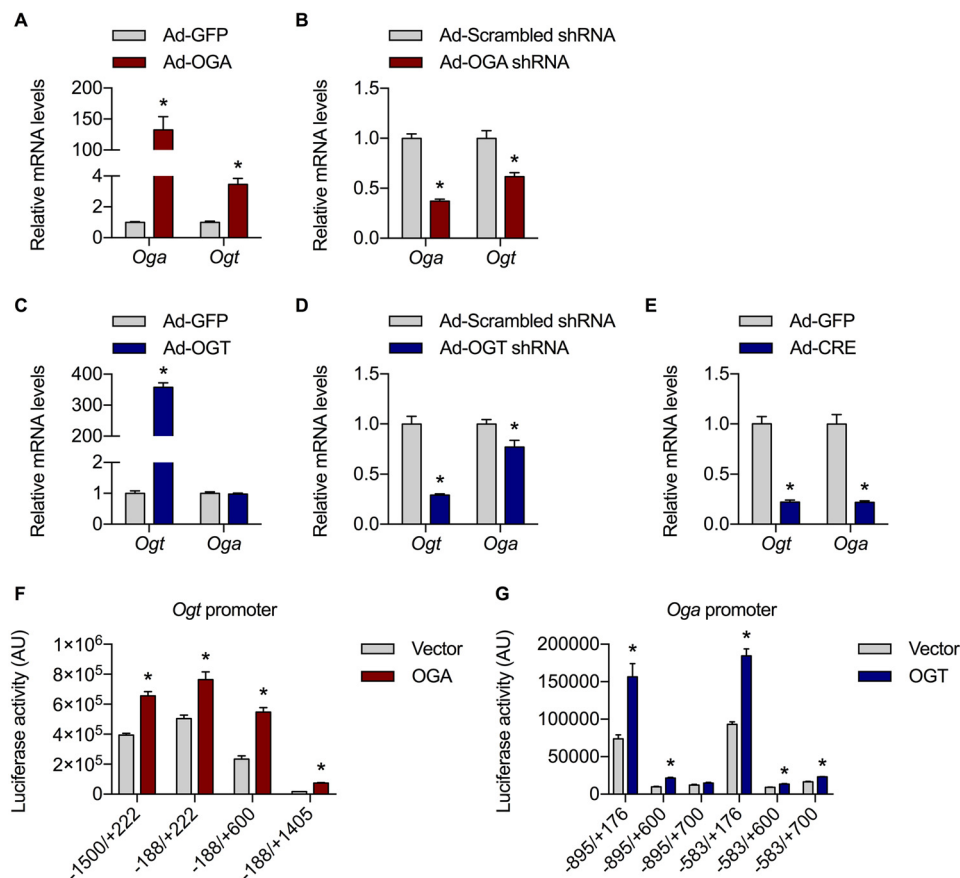


Figure 1. OGA regulates *Ogt* transcription and vice versa. A–D, primary hepatocytes isolated from WT mice were transduced with adenoviral vectors expressing GFP or OGA ($n = 12, 6$) (A), scrambled or OGA shRNA ($n = 6$) (B), GFP or OGT ($n = 12, 6$) (C), and scrambled or OGT shRNA ($n = 6$) (D), and *Oga* and *Ogt* mRNA levels were measured by RT–qPCR. E, primary hepatocytes isolated from *Ogt*-floxed mice were transduced with adenoviral vectors expressing GFP or CRE ($n = 6$), and *Ogt* and *Oga* mRNA levels were measured by RT–qPCR. F and G, *Ogt* ($n = 4$) (F) and *Oga* ($n = 4$) (G) promoter luciferase assays performed in CV-1 cells transfected with the indicated plasmids. All values represent means \pm S.E. *, $p < 0.05$ by multiple t tests with Holm–Sidak correction for multiple comparisons.

ferase p300, promotes *Ogt* transcription via the transcription factor CCAAT/enhancer-binding protein β (C/EBP β).

Although the role of aberrant protein O-GlcNAcylation in the pathogenesis of various human disorders has become increasingly well characterized, the molecular mechanisms responsible for altered cellular O-GlcNAc homeostasis in these conditions remain poorly studied. For instance, OGT and OGA expression levels are known to be elevated in numerous human cancers and both OGT and OGA have been shown to promote tumorigenesis (10, 19–25); however, how OGT and OGA expression levels are differentially regulated in cancer versus normal cells is a fundamental question that requires further investigation. Here, we show that OGT and OGA mRNA levels are highly correlated across a wide range of human cancers, particularly in pancreatic adenocarcinoma. Mechanistically, we demonstrate that the OGA–C/EBP β axis regulates *Ogt* expression in primary mouse pancreatic ductal adenocarcinoma (PDAC) cells. Furthermore, we show that loss of extracellular signal-regulated kinase (ERK) signaling in these cells decreases OGA glycosidase activity and reduces OGT mRNA and protein levels, raising the possibility that oncogenic ERK signaling may impinge upon OGA-mediated *Ogt* transcription to increase OGT expression in pancreatic cancer.

Results

OGA regulates *Ogt* transcription and vice versa

To test the hypothesis that OGT and OGA regulate each other at the transcriptional level, primary hepatocytes were first isolated from WT mice and transduced with adenoviral vectors expressing OGA, OGA shRNA, OGT, or OGT shRNA. Overexpression of OGA produced a concomitant increase in *Ogt* mRNA levels, whereas knockdown of OGA reduced *Ogt* expression (Fig. 1, A and B). Although overexpression of OGT failed to induce *Oga* expression, knockdown of OGT did result in a slight but significant decrease in *Oga* mRNA levels (Fig. 1, C and D). To determine whether knocking out OGT would produce a more dramatic change in *Oga* expression, primary hepatocytes were isolated from *Ogt*-floxed mice and transduced with an adenoviral vector expressing Cre. Deletion of the *Ogt* gene led to a dramatic reduction in both *Ogt* and *Oga* mRNA levels (Fig. 1E). Taken together, these data demonstrate that OGA modulates *Ogt* expression and vice versa.

To determine whether the observed changes in *Ogt* and *Oga* gene expression could be due to altered regulation of *Ogt* and *Oga* transcription, luciferase reporter constructs containing various truncations of the mouse *Ogt* and *Oga* promoters were generated. Overexpression of OGA in CV-1 cells transiently

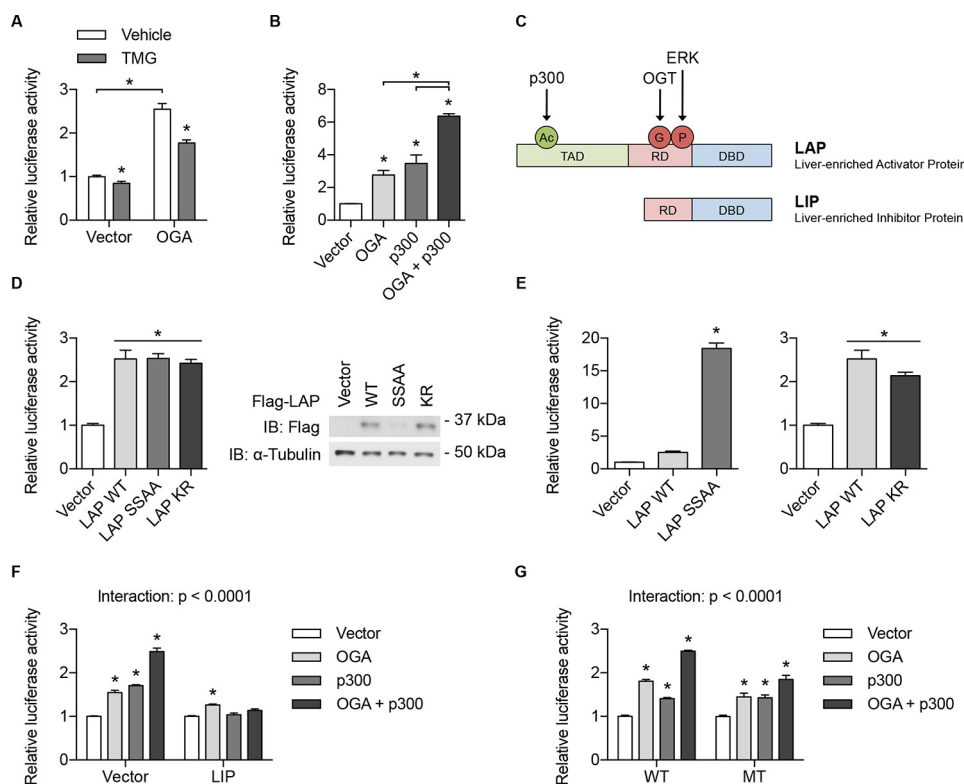


Figure 2. OGA and p300 promote *Ogt* transcription through C/EBP β . A, B, and D–G, *Ogt* promoter luciferase assays performed in HEK 293T cells transfected with the indicated plasmids. A, HEK 293T cells were also treated with vehicle or TMG (40 μ M, 24 h). D, right panel, Western blotting showing the expression level of each protein after transfection of equal amounts of DNA. See Fig. S1B for quantification. E, data from D after normalizing luciferase activity to the relative levels of LAP SSAA and LAP KR. G, luciferase assay was performed using both WT and C/EBP β -binding site mutation (MT) *Ogt* promoter luciferase reporter constructs. See Fig. S1, D and E for binding site sequence and mutation. All values represent means \pm S.E. ($n = 4$ for A and D–G and $n = 3$ for B). *, $p < 0.05$ by Student's *t* test (A, vector versus OGA), multiple *t* tests with Holm–Sidak correction for multiple comparisons (A, vehicle versus TMG), one-way analysis of variance with Tukey's multiple comparisons test (B, D, and E), or two-way analysis of variance with Dunnett's multiple comparisons test (F and G). C, schematic illustration of the domain structures and post-translational modifications of the two main isoforms of C/EBP β . TAD, transactivation domain; RD, regulatory domain; DBD, DNA-binding domain; Ac, acetylation; G, O-GlcNAcylation; P, phosphorylation; IB, immunoblot.

expressing any of the four *Ogt* promoter luciferase reporters resulted in a significant increase in *Ogt* promoter activity; similarly, overexpression of OGT induced *Oga* promoter activity in five of the six *Oga* promoter reporter constructs (Fig. 1, F and G). Collectively, these data demonstrate that OGA promotes *Ogt* transcription and vice versa and suggest that mutual regulation of OGT and OGA does indeed exist at the transcriptional level.

To corroborate these findings, the genes most highly coexpressed with OGT and OGA were obtained from the gene coexpression database COXPRESdb (26). This analysis revealed that OGA was among the top genes coexpressed with OGT in human, mouse, and rat tissues and vice versa (Table S1 and S2). The correlation was particularly notable in human tissues, where OGA was ranked ninth among OGT coexpressed genes, and OGT was ranked fifth among OGA coexpressed genes. These data further support the notion that OGT and OGA expression levels are tightly coupled and suggest that this homeostatic phenomenon is conserved across tissues and species.

OGA and p300 promote *Ogt* transcription through C/EBP β

Our next aim was to examine the molecular mechanisms underlying mutual regulation of OGT and OGA at the transcriptional level, with a focus on OGA-mediated *Ogt* transcrip-

tion because *Ogt* mRNA levels were particularly responsive to changes in *Oga* expression (Fig. 1, A and B). To probe this pathway, the luciferase reporter construct containing the $-1500/+222$ region of the mouse *Ogt* promoter was first transiently expressed in human embryonic kidney (HEK) 293T cells. Overexpression of OGA in these cells produced an increase in *Ogt* promoter activity that was blunted by concomitant administration of the OGA inhibitor Thiamet G (TMG), suggesting that OGA promotes *Ogt* transcription at least in part through its glycosidase activity (Fig. 2A) (27).

Because OGA is not known to possess a DNA-binding domain, it likely regulates *Ogt* transcription by functioning as a coactivator for specific transcription factors (2, 4, 5). To identify candidate coactivators and transcription factors that may regulate *Ogt* transcription in conjunction with OGA, ChIP sequencing (ChIP-seq) data from the Encyclopedia of DNA Elements (ENCODE) were visualized in the University of California, Santa Cruz Genome Browser. These data revealed that the histone acetyltransferase and transcriptional coactivator p300 is localized to the human OGT promoter (Fig. S1A). Additionally, the OGT promoter was found to be enriched in histone H3 lysine 27 acetylation (H3K27ac) (Fig. S1A), an active promoter and enhancer mark deposited by the p300/CBP coactivator family (28–30). Consistent with these observations, coexpres-

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sion of OGA and p300 induced *Ogt* promoter activity to a greater extent than did overexpression of either OGA or p300 alone (Fig. 2B).

Analysis of ENCODE ChIP-seq data also identified C/EBP β as one of the most abundant transcription factors at the human *OGT* promoter (Fig. S1A). Previous studies have shown that O-GlcNAcylation of C/EBP β at serines 180 and 181 blocks its DNA binding and that acetylation of C/EBP β at lysine 39 by p300 enhances its transcriptional activity, suggesting that OGA and p300 could function as coactivators for C/EBP β (Fig. 2C) (31, 32). To determine whether C/EBP β promotes *Ogt* transcription, liver-enriched activator protein (LAP), one of the two main isoforms of C/EBP β (Fig. 2C), was overexpressed in HEK 293T cells transiently expressing the *Ogt* promoter luciferase reporter (31). Overexpression of wildtype LAP (LAP WT) increased *Ogt* promoter activity, indicating that C/EBP β does indeed promote *Ogt* transcription (Fig. 2D). Mutation of the LAP O-GlcNAcylation sites to alanine (LAP SSAA) or the lysine 39 acetylation site to arginine (LAP KR) initially appeared to have no effect on the ability of LAP to regulate *Ogt* promoter activity; however, Western blotting analysis revealed that LAP SSAA levels were markedly lower and LAP KR levels were slightly higher than those of LAP WT despite transfection of equal amounts of DNA (Fig. 2D and Fig. S1B). This observation could be explained by compensatory degradation and stabilization of highly active (LAP SSAA) and less active (LAP KR) transcription factors, respectively (33). Normalization of luciferase activity to the relative levels of LAP SSAA and LAP KR demonstrated that LAP SSAA was dramatically more active and LAP KR was slightly but not significantly less active than LAP WT at the *Ogt* promoter (Fig. 2E). Because coexpression of LAP and p300 increased *Ogt* promoter activity in a cooperative fashion (Fig. S1C), the marginal effect of the KR mutation even after normalization could be attributed to the existence of other LAP acetylation sites that contribute to its transcriptional activity. Alternatively, p300 may stimulate LAP-dependent *Ogt* transcription through acetylation-independent mechanisms (34). Taken together, these data suggest that OGA and p300 regulate *Ogt* transcription in conjunction with C/EBP β , perhaps by reducing its O-GlcNAcylation and increasing its acetylation.

To determine whether C/EBP β is required for OGA- and p300-mediated regulation of *Ogt* transcription, liver-enriched inhibitor protein (LIP), the dominant negative isoform of C/EBP β (Fig. 2C), was coexpressed with OGA and p300 in HEK 293T cells transiently expressing the *Ogt* promoter luciferase reporter (31, 35). Overexpression of LIP almost completely abolished the effect of OGA and/or p300 on *Ogt* promoter activity, indicating that LAP activity is essential for OGA- and p300-dependent *Ogt* transcription (Fig. 2F). To corroborate these results, we next sought to determine whether mutating the C/EBP β -binding site on the *Ogt* promoter affects the ability of OGA and p300 to promote *Ogt* transcription. To address this question, the DNA sequence of the mapped C/EBP β -binding region was first obtained from the University of California, Santa Cruz Genome Browser (Fig. S1A) and searched for the C/EBP β consensus sequence 5'-T[TG]NNGNAA[TG]-3' (36). One DNA element matching the consensus sequence was identified and found to be conserved among the human, mouse, and

rat *OGT* promoters (Fig. S1D). Two consecutive bases of the identified binding motif were then mutated in the *Ogt* promoter luciferase reporter construct (Fig. S1E), which was subsequently expressed in HEK 293T cells with additional coexpression of OGA and p300. Mutation of the conserved C/EBP β -binding site significantly blunted the effect of OGA and/or p300 on *Ogt* promoter activity, suggesting that OGA- and p300-mediated *Ogt* transcription is at least partially dependent on C/EBP β binding to the *Ogt* promoter (Fig. 2G). The incomplete abrogation of *Ogt* promoter activity by the C/EBP β -binding site mutation could be attributed to the existence of other C/EBP β -binding sites on the *Ogt* promoter, to residual binding of C/EBP β to the mutated binding site, or to the unperturbed actions of other transcription factors. Collectively, these data demonstrate that OGA and p300 promote *Ogt* transcription through C/EBP β .

OGT and OGA expression levels are highly correlated in human cancers

Thus far, we have demonstrated the existence of mutual regulation of OGT and OGA at the transcriptional level and have outlined a potential mechanism that may contribute to this phenomenon in a physiologic system. Because perturbations in cellular O-GlcNAc homeostasis have been linked to numerous human disorders, our next aim was to examine the role of mutual regulation of OGT and OGA in the pathogenesis of a relevant human disease. Altered expression of OGT and OGA has been observed in a wide range of human cancers; thus, we postulated that cancer would be an appropriate system in which to study the relevance of mutual regulation of OGT and OGA to human disease.

To broadly assess the potential role of mutual regulation of OGT and OGA in cancer pathophysiology, The Cancer Genome Atlas (TCGA) gene expression data for 32 types of human cancer were analyzed for correlations between *OGT* and *OGA* expression (37, 38). Remarkably, a significant positive correlation between *OGT* and *OGA* mRNA levels was observed in nearly every cancer type analyzed, including the four most prevalent cancers in the United States: breast, colorectal, lung, and prostate (Fig. 3, A–D, and Table S3) (39). Further analysis of TCGA data revealed that genomic alterations (*i.e.* amplifications, deletions, and/or mutations) in the *OGT* and *OGA* genes are relatively rare in human cancers, suggesting that dysregulation of *OGT* and *OGA* expression in cancer cells may be driven primarily by transcriptional rather than genomic changes (Fig. S2, A and B). These data demonstrate that *OGT* and *OGA* expression levels are highly correlated in human cancers and suggest that altered transcriptional regulation of *OGT* and *OGA* may play a particularly important role in cancer biology.

OGT and OGA expression levels are highly correlated and elevated in human pancreatic cancer

Among the cancer types with the highest correlation coefficients (Fig. 3E), pancreatic adenocarcinoma was selected for further analysis because perturbations in O-GlcNAc homeostasis had previously been described in this form of cancer (40–42). In addition to a strong positive correlation between *OGT* and *OGA* expression, pancreatic adenocarcinoma was found to

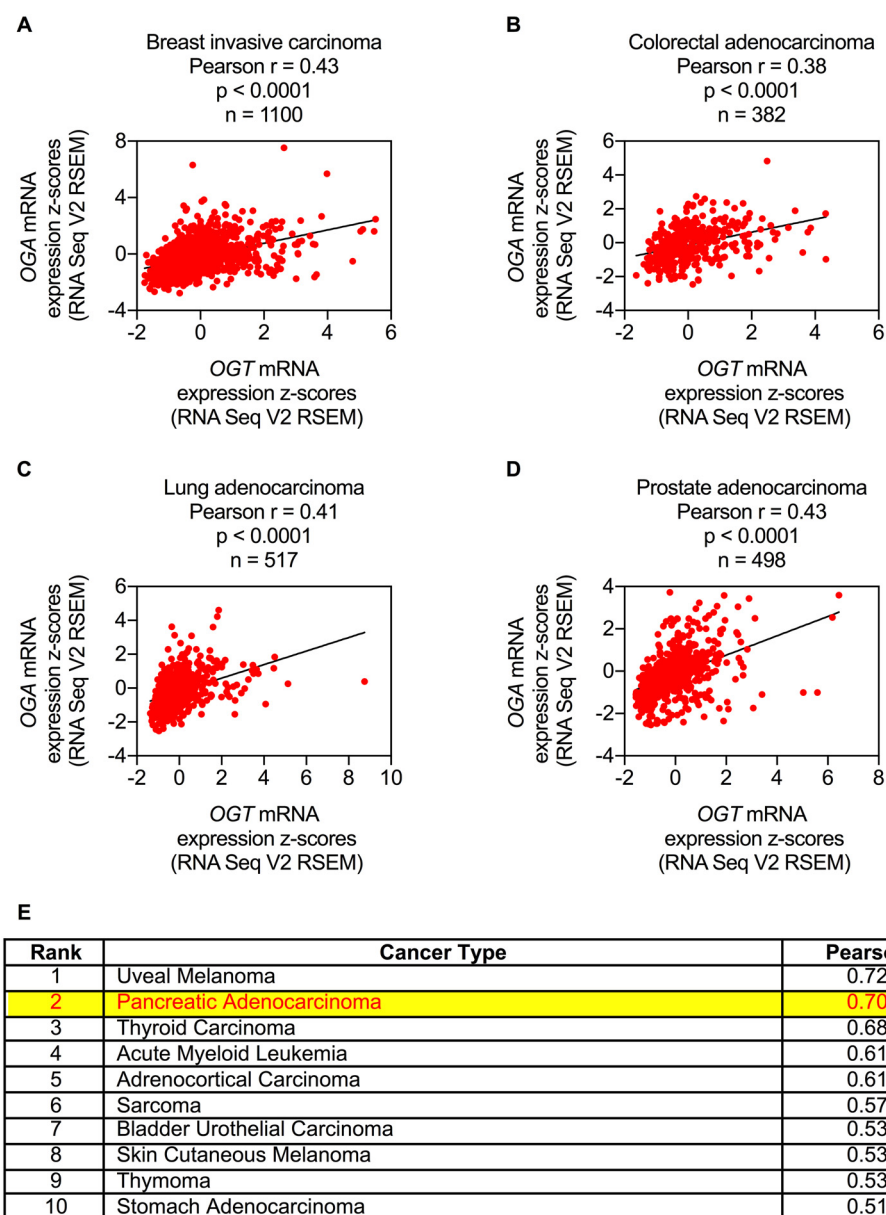


Figure 3. *OGT* and *OGA* expression levels are highly correlated in human cancers. A–D, *OGT* versus *OGA* mRNA expression z scores from TCGA RNA Seq V2 RSEM data for breast invasive carcinoma (A), colorectal adenocarcinoma (B), lung adenocarcinoma (C), and prostate adenocarcinoma (D). E, ranked list of cancer types with the highest Pearson correlation coefficients for *OGT* versus *OGA* mRNA expression z scores. See Table S3 for complete list.

have elevated *OGT* and *OGA* mRNA levels relative to normal pancreas (Fig. 4, A and B). To determine whether these transcript-level phenomena were also observable at the protein level, tissue sections of normal human pancreas and PDAC were stained for *OGT*, *OGA*, and *O*-GlcNAc. These immunohistochemical analyses revealed that *OGT*, *OGA*, and protein *O*-GlcNAcylation levels are dramatically elevated in PDAC relative to normal pancreas (Fig. 4, C and D, and Table S4). Notably, significant positive correlations among *OGT*, *OGA*, and *O*-GlcNAc were observed in PDAC, including an unexpected positive correlation between *OGA* and *O*-GlcNAc (Fig. 4E and Tables S5–S7). Taken together, these data demonstrate that both mRNA and protein levels of *OGT* and *OGA* are highly correlated and elevated in human pancreatic cancer and indicate that this expression pattern is ultimately associated with increased protein *O*-GlcNAcylation.

OGA, *C/EBPβ*, and *ERK* signaling regulate *Ogt* expression in pancreatic cancer

Our next aim was to examine the potential mechanisms leading to correlation and elevation of *OGT* and *OGA* expression levels in pancreatic cancer. We hypothesized that 1) mutual regulation of *OGT* and *OGA* at the transcriptional level remains largely intact in pancreatic cancer cells, leading to a positive correlation between *OGT* and *OGA* expression, and 2) an aberrantly activated signaling pathway in these cells impinges upon mutual regulation of *OGT* and *OGA* to increase the expression of these genes.

To test these hypotheses in an established *in vitro* system, primary mouse PDAC cells with doxycycline-inducible expression of *Kras*^{G12D} and hemizygous deletion of *p53* were first transduced with *OGA* shRNA adenovirus or treated with TMG

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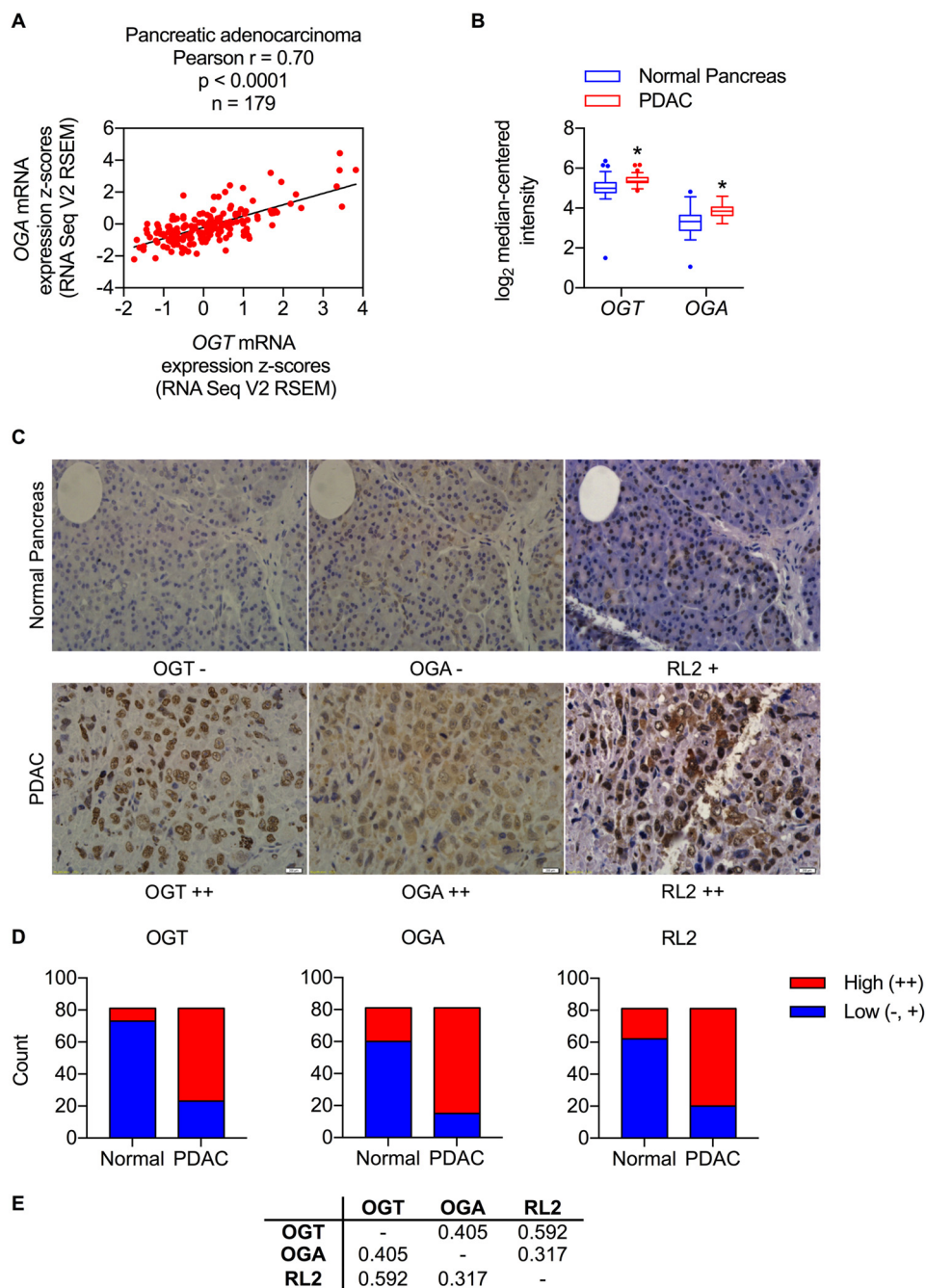


Figure 4. OGT and OGA expression levels are highly correlated and elevated in human pancreatic cancer. *A*, OGT versus OGA mRNA expression z scores from TCGA RNA Seq V2 RSEM data for pancreatic adenocarcinoma. *B*, box-and-whisker plot of OGT and OGA mRNA expression levels in normal pancreas versus PDAC ($n = 39$). *, $p < 0.05$ by multiple t tests with Holm–Sidak correction for multiple comparisons. *C*, representative images of normal pancreas and PDAC tissue sections stained for OGT, OGA, and RL2 (O-GlcNAc). *D*, counts of normal pancreas and PDAC tissue sections with low or high levels of OGT, OGA, and RL2 staining ($n = 81$). See Table S4 for raw data and statistical analysis. *E*, table of Spearman correlation coefficients for OGT versus OGA, OGT versus RL2, and OGA versus RL2 staining levels in PDAC. See Tables S5–S7 for raw data and statistical analyses.

(40). Genetic knockdown of OGA and pharmacological inhibition of OGA glycosidase activity both reduced *Ogt* mRNA levels in these cells, suggesting that OGA also regulates *Ogt* transcription in pancreatic cancer (Fig. 5, *A* and *B*). Interestingly, TMG treatment elicited a compensatory increase in *Oga* mRNA levels, indicating that *Oga* expression is also responsive to changes in O-GlcNAc homeostasis as reported previously (Fig. 5*B*) (15). Consistent with a role for C/EBP β in modulating *Ogt* transcription in pancreatic cancer, C/EBP β occupancy was found to be

highly and specifically enriched at the *Ogt* promoter in PDAC cells and overexpression of LIP led to a significant decrease in *Ogt* mRNA levels (Fig. 5, *C* and *D*). Taken together, these data demonstrate that the OGA–C/EBP β axis also regulates *Ogt* expression in PDAC cells, supporting our first hypothesis that mutual regulation of OGT and OGA at the transcriptional level remains largely intact in pancreatic cancer.

Over 90% of human PDACs possess gain-of-function mutations in the *KRAS* oncogene, which lead to constitutive

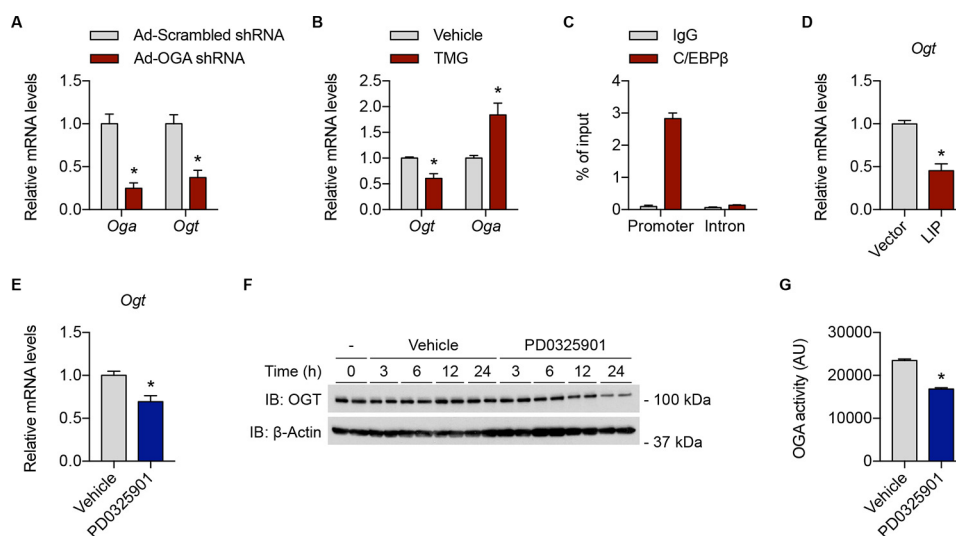


Figure 5. OGA, C/EBP β , and ERK signaling regulate *Ogt* expression in pancreatic cancer. *A*, primary mouse PDAC cells were transduced with adenoviral vectors expressing scrambled or OGA shRNA, and *Oga* and *Ogt* mRNA levels were measured by RT–qPCR. *B*, primary mouse PDAC cells were treated with vehicle or TMG (10 μ M, 12 h), and *Ogt* and *Oga* mRNA levels were measured by RT–qPCR. *C*, sheared chromatin from primary mouse PDAC cells was immunoprecipitated with IgG or an anti-C/EBP β antibody and analyzed by RT–qPCR. Promoter primers amplify a 124-bp region containing the C/EBP β binding site in Fig. S1D, and intron primers target a 73-bp region in intron 11–12. *D*, primary mouse PDAC cells were transfected with vector or LIP and *Ogt* mRNA levels were measured by RT–qPCR. *E–G*, primary mouse PDAC cells were treated with vehicle or PD0325901 (10 μ M) for 12 h (*E*); 0, 3, 6, 12, or 24 h (*F*); or 6 h (*G*), and *Ogt* mRNA levels were measured by RT–qPCR (*E*), OGT protein levels were measured by Western blotting (*F*), or total cellular OGA activity was measured by OGA activity assay (*G*). See Fig. S3B for quantification of *F*. All values represent means \pm S.E. ($n = 3$ for *A–D* and *G*; $n = 6$ for *E*, pooled from two independent experiments). *, $p < 0.05$ by multiple t tests with Holm–Sidak correction for multiple comparisons (*A* and *B*) or Student’s t test (*D*, *E*, and *G*). *IB*, immunoblot.

activation of downstream signaling pathways such as the mitogen-activated protein kinase (MAPK)/ERK pathway. Because transgenic *Kras*^{G12D} expression had previously been shown to raise protein O-GlcNAcylation levels in PDAC cells, we hypothesized that aberrant activation of ERK signaling in these cells impinges upon OGA-mediated *Ogt* transcription to increase OGT expression (40). To test this hypothesis, ERK signaling was inhibited in PDAC cells using PD0325901 (Fig. S3A), a potent and selective inhibitor of the upstream ERK activator MAPK/ERK kinase (MEK) (43, 44). Inhibition of MEK led to a significant decrease in both mRNA and protein levels of OGT, indicating that ERK signaling regulates OGT expression in PDAC cells (Fig. 5, *E* and *F*, and Fig. S3B). Because OGA-mediated *Ogt* transcription is at least partially dependent on OGA glycosidase activity (Fig. 2A), we next sought to determine whether ERK signaling also modulates OGA activity in PDAC cells. Indeed, inhibition of MEK using PD0325901 reduced total cellular OGA activity without altering OGA protein levels (Fig. 5G and Fig. S3C). Collectively, these data suggest that oncogenic ERK signaling may up-regulate OGT expression in pancreatic cancer by increasing OGA glycosidase activity.

Discussion

Aberrant protein O-GlcNAcylation is being identified as a key pathological feature of an increasing number of human disorders; thus, elucidating the mechanisms through which cellular O-GlcNAc homeostasis is maintained in the physiologic state and altered in the disease state is of significant biomedical interest. We previously hypothesized that cellular O-GlcNAcylation levels are maintained within an optimal zone by mutual regulation of the O-GlcNAc–cycling enzymes OGT and OGA (2). Here, using established *in vitro* systems and large-scale analysis of human cancer data, we demonstrate that 1)

OGT and OGA indeed regulate each other at the transcriptional level; 2) OGA promotes *Ogt* transcription through cooperation with the histone acetyltransferase p300 and transcription factor C/EBP β ; 3) OGT and OGA expression levels are highly correlated across a wide range of human cancers, particularly in pancreatic adenocarcinoma; and 4) OGA, C/EBP β , and ERK signaling regulate *Ogt* expression in PDAC.

To demonstrate the existence of mutual regulation of OGT and OGA at the transcriptional level, we used both genetic and pharmacological methods to manipulate OGT and OGA in a variety of cell types, including primary mouse hepatocytes, CV-1 monkey kidney cells, HEK 293T cells, and primary mouse PDAC cells. One important question for future investigation is whether mutual regulation of OGT and OGA at the transcriptional level is a universal phenomenon that exists in all cell types of an organism. Numerous studies in diverse cellular contexts have reported compensatory changes in OGT and OGA mRNA levels in response to manipulation of the O-GlcNAc and hexosamine biosynthetic pathways, suggesting that mutual regulation of OGT and OGA at the transcriptional level may indeed be a fundamental process that is generalizable to many cell types (15, 16, 45–47). However, in contrast to our findings in primary hepatocytes and PDAC cells, Zhang *et al.* (15) demonstrated in three different cell lines (HeLa cervical adenocarcinoma, K562 chronic myelogenous leukemia, and SH-SY5Y neuroblastoma cells) that OGA but not OGT expression is responsive to manipulation of O-GlcNAc signaling, indicating that the specific mechanisms underlying this process (*e.g.* the OGA–p300–C/EBP β axis described here) likely differ among cell types. We speculate that cell type–specific genetic programs ultimately determine the precise mechanisms controlling mutual regulation of OGT and OGA in different cellular contexts.

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Mutual regulation of OGT and OGA may also occur at levels beyond transcription. For instance, Park *et al.* (16) identified a post-transcriptional mechanism that differentially regulates splicing of *OGT* pre-mRNA in response to changes in O-GlcNAc homeostasis. In this pathway, high cellular O-GlcNAcylation levels promote *OGT* intron retention and subsequent mRNA degradation, whereas low cellular O-GlcNAcylation levels enhance the formation of fully spliced *OGT* mRNA. Intriguingly, deletion of the *OGT* intronic splicing silencer in colon cancer cells increases the expression of both OGT and OGA and renders these cells highly sensitive to TMG treatment, suggesting that compensatory suppression of OGT expression in response to OGA inhibition is essential for cell survival (16). In future studies, it would be interesting to determine whether OGA plays a direct role in regulating *OGT* pre-mRNA splicing and intron retention as it does in modulating *Ogt* transcription. In addition to transcriptional and post-transcriptional mechanisms, mutual regulation of OGT and OGA may also occur at the post-translational level because OGT and OGA have themselves been identified as O-GlcNAcylated proteins (17, 18). Post-translational regulation of OGT and OGA activity and/or stability would allow cells to respond more rapidly to perturbations in O-GlcNAc homeostasis than would transcriptional or post-transcriptional regulation of *OGT* and *OGA* expression; thus, elucidating these post-translational mechanisms will be an important area for future investigation in the field.

In addition to mutual regulation of OGT and OGA, transcription factors that coregulate *OGT* and *OGA* expression may also contribute to maintenance of O-GlcNAc homeostasis. For instance, Muthusamy *et al.* (48) demonstrated that the transcription factor E2F1 binds to the promoters of both *OGT* and *OGA* and suppresses their expression, raising the possibility that E2F1 can concurrently repress *OGT* and *OGA* transcription to maintain a balance between *OGT* and *OGA* mRNA levels. Notably, ENCODE ChIP-seq analysis also identified E2F1 as one of the most abundant transcription factors at the human *OGT* promoter (Fig. S1A).

By analyzing TCGA gene expression data, we found that *OGT* and *OGA* mRNA levels are highly correlated across a wide range of human cancers. We then used pancreatic cancer as a model to demonstrate that oncogenic signaling pathways such as the MAPK/ERK pathway have the potential to impinge upon OGA-mediated *Ogt* transcription to increase OGT expression. One important question for future investigation is whether ERK-dependent regulation of OGT expression is a feature of cancer types other than pancreatic adenocarcinoma, which may be particularly reliant on ERK signaling because >90% possess gain-of-function mutations in *KRAS* (40). Intriguingly, many of the other cancer types with the highest correlation coefficients for *OGT* versus *OGA* expression are also characterized by frequent activating mutations in the MAPK/ERK signaling pathway (e.g. thyroid carcinoma, skin cutaneous melanoma) (Fig. 3E) (49, 50). Furthermore, a previous study by Zhang *et al.* (51) demonstrated that ERK signaling also stimulates OGT expression in lung and prostate cancer cells. These findings suggest that ERK-dependent regulation of OGT expression is

not limited to pancreatic adenocarcinoma and may in fact be a feature of a wide range of human cancers.

Because inhibition of MEK reduces total cellular OGA activity in PDAC cells, we propose that ERK signaling regulates OGT expression at least in part by modulating OGA glycosidase activity. In future studies, it will be important to determine whether ERK regulates OGA activity through direct or indirect mechanisms. Interestingly, a chemical genetic screen for ERK2 substrates based on engineered analog-sensitive kinases identified OGA as a novel target of ERK2, raising the possibility that ERK can modulate OGA activity through direct phosphorylation (52). Alternative mechanisms through which ERK signaling may regulate OGT expression include modulation of C/EBP β DNA-binding activity. Indeed, removal of O-GlcNAc from serines 180 and 181 of C/EBP β leads to phosphorylation of neighboring threonine 188 by ERK, which in turn promotes C/EBP β DNA binding (31, 53). Thus, OGA and ERK may cooperatively stimulate *OGT* transcription by modulating the interplay between O-GlcNAcylation and phosphorylation on the regulatory domain of C/EBP β . Attenuation of oncogenic ERK signaling in PDAC cells via loss of *Kras*^{G12D} transgene expression has also been shown to reduce cellular O-GlcNAcylation levels by decreasing *Gfpt1* expression, indicating that ERK signaling can alter O-GlcNAc homeostasis in PDAC through a variety of different mechanisms (40).

Increases in OGT expression and decreases in OGA expression have been reported in a number of human cancers, putting forth the idea that uncoupling of OGT and OGA expression is responsible for elevated O-GlcNAcylation levels in cancer cells (21, 24, 41). However, in the present study and our previous work, we utilized large-scale analysis of human cancer data to show that OGT and OGA levels are in fact positively correlated and concomitantly up-regulated in most human cancer types.⁴ We also demonstrate here that the OGA–C/EBP β axis that regulates *Ogt* transcription in normal cells appears to be retained in PDAC cells. These results raise an important question: how are O-GlcNAcylation levels increased in cancer cells if both OGT and OGA levels are elevated? Our previous work suggests that, under high-glucose conditions, OGA may in fact promote the O-GlcNAcylation of specific substrates via its cryptic acetyltransferase activity, raising the possibility that OGT and OGA can act cooperatively on key proteins to promote cell proliferation.⁴ Consistent with this hypothesis, OGA overexpression enhances tumor growth in a xenograft model,⁴ and partial loss of OGA suppresses intestinal tumorigenesis in *Apc*^{min/+} mice (25). Thus, we propose that mutual regulation of OGT and OGA at the transcriptional level serves to maintain O-GlcNAc homeostasis in normal cells but can be utilized by cancer cells to drive increased expression of both OGT and OGA and thereby promote tumor growth.

Experimental procedures

Cell culture

Primary hepatocytes were isolated from WT and *Ogt*-floxed C57BL/6 mice by the Yale Liver Center Core Facility and cul-

⁴ K. Qian, S. Wang, M. Fu, J. Zhou, J. P. Singh, M.-D. Li, Y. Yang, K. Zhang, J. Wu, Y. Nie, H.-B. Ruan, and X. Yang, unpublished results.

tured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS), 2 mM sodium pyruvate, 1 μ M dexamethasone, and 0.1 μ M insulin on collagen I-coated plates. CV-1 and HEK 293T cells were cultured in Dulbecco's modified Eagle's medium with 10% FBS. PDAC cells were generously provided by Dr. Alec Kimmelman (New York University) and cultured in RPMI 1640 medium with 10% FBS and 1 μ g/ml doxycycline to maintain *Kras*^{G12D} transgene expression (40). The cells were transfected using FuGENE HD transfection reagent (Promega). The cells were treated with TMG (Carbosynth) and PD0325901 (Sigma) as indicated.

Adenoviruses and plasmids

Recombinant adenoviruses were generated using the AdEasy system (54). *Ogt* and *Oga* promoter luciferase reporter constructs were generated by cloning the indicated regions of the mouse *Ogt* and *Oga* promoters into the pGL3-Basic luciferase reporter vector (Promega). OGT and OGA expression vectors were generated by cloning FLAG/HA-rOGT and 3*FLAG/2*Myc-mOGA into the pAdTrack-CMV plasmid. pCMV β -p300-myc (catalog no. 30489) and pCMV-FLAG-LIP (catalog no. 15737) plasmids were purchased from Addgene (55). pcDNA3.1-FLAG-LAP WT-HA and pcDNA3.1-FLAG-LAP SSAA-HA plasmids were generously provided by Dr. Qi-Qun Tang (Fudan University) (31). Plasmids with point mutations (pcDNA3.1-FLAG-LAP KR-HA and MT *Ogt* promoter luciferase reporter construct) were generated using the QuikChange XL II site-directed mutagenesis kit (Agilent).

Quantitative reverse transcription-PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen). cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). cDNA was amplified and quantified using iQ SYBR Green Supermix (Bio-Rad) and the LightCycler 480 system (Roche). All data were normalized to *36b4* expression. The primer sequences were as follows: *36b4*-F, 5'-AGA TGC AGC AGA TCC GCA T-3'; *36b4*-R, 5'-GTT CTT GCC CAT CAG CAC C-3'; *Ogt*-F, 5'-AAG AGG CAC GCA TTT TTG AC-3'; *Ogt*-R, 5'-ATG GGG TTG CAG TTC GAT AG-3'; *Oga*-F, 5'-CTC AGA GGC TGA GAA AAT AAT GTT GAG-3'; and *Oga*-R, 5'-AAG GGA AGT TGG CAA GGA AAG T-3'.

Luciferase assay

The cells were seeded in 48-well plates for 24 h and then transfected with 100 ng of each plasmid (luciferase reporter construct and expression vectors). After 48 h, the cells were lysed using passive lysis buffer (Promega), and luciferase assay was performed according to the manufacturer's protocol.

Western blotting

Anti-FLAG (F3165), anti- α -tubulin (T5168), and anti- β -actin (A5441) antibodies were purchased from Sigma. Anti-OGT (ab96718) and anti- β -tubulin (ab15568) antibodies were purchased from Abcam. Anti-p-ERK (9106) and anti-ERK (9102) antibodies were purchased from Cell Signaling. Anti-OGA (NBP1-96679) antibody was purchased from Novus. The cells were lysed in passive lysis buffer (Promega) or 1% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1 mM EDTA, and

protease and phosphatase inhibitors. Equal amounts of whole cell lysate were electrophoresed on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Membranes were incubated with primary antibodies overnight at 4 °C. Western blotting visualization was performed using horseradish peroxidase-conjugated secondary antibodies and ECL substrate. Western blotting quantification was performed using Adobe Photoshop.

Chromatin immunoprecipitation

ChIP was performed as described previously (56, 57). Briefly, the cells grown to confluence in 10-cm dishes were cross-linked with 1% formaldehyde for 8 min and quenched with glycine (final concentration, 125 mM). The cells were then lysed and sonicated using a Misonix Sonicator 3000 (20 cycles, 7 s on, 45 s off, output level 5). Sheared chromatin was immunoprecipitated by overnight incubation with an anti-C/EBP β antibody (sc-150, Santa Cruz), and antibody-chromatin complexes were captured by a 1-h incubation with protein A/G beads (sc-2003, Santa Cruz). Immunoprecipitated and input chromatin was de-cross-linked overnight and purified by phenol-chloroform extraction and ethanol precipitation. Chromatin samples were then analyzed by qPCR. All data were normalized to input DNA. Promoter primers amplify a 124-bp region containing the C/EBP β -binding site in Fig. S1D, and intron primers target a 73-bp region in intron 11-12. The primer sequences were as follows: promoter-F, 5'-CCT AGA CAG GGT TGT CGC AT-3'; promoter-R, 5'-GCG CGT AAC AAG ACT ACC GA-3'; intron-F, 5'-TCG TTG TCA GGT ACG CAA GG-3'; and intron-R, 5'-AGG CAA TCT CCT ACA AGC ATG G-3'.

OGA activity assay

OGA activity assay was performed as described previously (58). Briefly, duplicate 50- μ l reactions containing whole cell lysate, 50 mM sodium cacodylate, pH 6.4, 0.3% BSA, 100 mM *N*-acetyl-D-galactosamine (GalNAc), and 1 mM 4-methylumbelliferyl (4MU)-GlcNAc or 4MU-GalNAc (Sigma) were set up in black flat-bottomed 96-well plates. The reactions were incubated at 37 °C for 1–2 h and quenched with glycine, pH 10.75 (final concentration, 150 mM). Fluorescence intensity was measured using a Molecular Devices Spectramax M5 microplate reader (excitation, 368 nm; emission, 450 nm; sensitivity, 50). OGA activity was calculated by subtracting 4MU-GalNAc fluorescence from 4MU-GlcNAc fluorescence to exclude lysosomal hexoaminidase activity.

Gene expression data set analysis

Analysis of top genes coexpressed with *OGT* or *OGA* in human, mouse, and rat tissues was performed using COXPRESdb (26). TCGA gene expression and genomic alteration data were analyzed using cBioPortal for Cancer Genomics (37, 38). Normal pancreas *versus* PDAC gene expression data were analyzed using OncoPrint (Thermo Fisher).

Immunohistochemistry

After baking on a panel at 70 °C, formalin-fixed paraffin-embedded tissue samples were deparaffinized with xylenes and

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rehydrated through gradient ethanol immersion. Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide in methanol for 12 min, followed by three 3-min washes with PBS. The sections were then blocked with 10% normal goat serum in PBS for 25 min, followed by overnight incubation with primary antibody in a moist chamber at 4 °C. Primary antibodies were diluted in PBS containing 1% BSA and preimmune serum was substituted for primary antibody as a negative control. After three 5-min washes with PBS, the sections were incubated with secondary antibody for 25 min at room temperature, followed by three additional 5-min washes with PBS. Reaction products were visualized with diaminobenzidine (ZLI-9032, ZSGB) at room temperature. After being counterstained with Harris hematoxylin (ZLI-9039, ZSGB) for 4 min and rinsed with tap water, the sections were immediately dehydrated by sequential immersion in gradient ethanol and xylenes and mounted with resin and cover slips. Images were obtained using an Olympus BX51 light microscope equipped with a DP70 digital camera. For evaluation of cell staining, sections were examined by two independent observers without prior knowledge of the clinical or clinicopathological status of the specimens. Antigen expression levels were stratified into negative (-), low (+), or high (++) by optical evaluation. Staining of less than 10% of the cancer cells in a core was classified as negative.

Statistical analysis

Statistical analysis was performed as indicated in the figure legends using GraphPad Prism 7.

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