Differential Regulation of the Ten-Eleven Translocation (TET) Family of Dioxygenases by O-Linked β-N-Acetylglucosamine Transferase (OGT)*

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Background: TET proteins have been shown to target OGT to chromatin, but whether OGT regulates the TET proteins is not clear.

Results: OGT regulates the subcellular localization and enzymatic activity of TET3 but not TET1 and TET2.

Conclusion: The TET family of proteins is differentially regulated by OGT.

Significance: We reveal a potential mechanism by which glucose metabolism regulates TET3 activity.

The ten-eleven translocation (TET) family of dioxygenases (TET1/2/3) converts 5-methylcytosine to 5-hydroxymethylcytosine and provides a vital mechanism for DNA demethylation. However, how TET proteins are regulated is largely unknown. Here we report that the O-linked β-GlcNAc (O-GlcNAc) transferase (OGT) is not only a major TET3-interacting protein but also regulates TET3 subcellular localization and enzymatic activity. OGT catalyzes the O-GlcNAcylation of TET3, promotes TET3 nuclear export, and, consequently, inhibits the formation of 5-hydroxymethylcytosine catalyzed by TET3. Although TET1 and TET2 also interact with and can be O-GlcNAcylated by OGT, neither their subcellular localization nor their enzymatic activity are affected by OGT. Furthermore, we show that the nuclear localization and O-GlcNAcylation of TET3 are regulated by glucose metabolism. Our study reveals the differential regulation of TET family proteins by OGT and a novel link between glucose metabolism and DNA epigenetic modification.

DNA methylation at the 5 position of cytosine (5mC) in the context of CpG dinucleotides is a major epigenetic modification in the mammalian genome that is critical for various biological and pathological processes (1, 2). As an epigenetic modification, DNA methylation has to be dynamic. Although the DNA methyltransferases that catalyze DNA methylation have been well studied (3, 4), less is known for DNA demethylation. In principle, DNA demethylation can be achieved through a replication-dependent passive mechanism involving inhibition of DNA maintenance methylation by DNMT1 during DNA replication and subsequent dilution through one or more rounds of mitosis (5). Alternatively, DNA demethylation can be achieved through a replication-independent active mechanism that involves the enzymes removing the methyl group from 5mC or the enzymes converting 5mC to other forms of modified bases that can be subsequently removed from DNA by DNA damage repair pathways (5, 6).

The recent discovery of the ten-eleven translocation (TET) family of dioxygenases has sparked great interest in DNA-active demethylation (7, 8). All three TET proteins (Tet1/2/3) have the capacity to convert 5mC to 5-hydroxymethylcytosine (5hmC) in a 2-oxoglutarate- and Fe(II)-dependent manner (9). 5hmC itself may function as a stable epigenetic mark or is further oxidized by TET proteins to form 5-formyl cytosine and 5-carboxylcytosine (10, 11). These derivative bases of 5hmC can be recognized by thymine DNA glycosylase, suggesting that TET proteins are able to initiate DNA demethylation through the base excision repair pathway (10, 12). In addition, because 5hmC is a poor template for DNMT1 (13), TET proteins can also lead to DNA demethylation through the replication-dependent passive mechanism.

The TET proteins are differentially expressed and exhibit both redundant and distinct functions (14, 15). For example, TET1 and TET2 are highly expressed in embryonic stem (ES) cells and Purkinje neurons (7–9), whereas TET3 is highly expressed in zygotes and plays a key role in selective demethylation of paternal DNA after fertilization (15, 16). In ES cells, TET1 has been shown to have dual roles in transcriptional activation and repression (17, 18), presumably through its 5hmC activity-dependent and independent function.

To better understand the regulation and function of TET proteins, various laboratories have carried out purification and characterization of TET-associated proteins. These efforts have led to identification of O-linked GlcNAc-transferase (OGT) as...
a protein associated with all three TET proteins (19–22). OGT transfers O-GlcNAc from UDP-GlcNAc to the hydroxyl group of serine or threonine residues of nuclear and cytoplasmic proteins (23, 24). OGT catalyzes O-GlcNAcylation of a broad range of substrates, whereas O-GlcNAcase (OGA) catalyzes the reverse reaction to remove the O-GlcNAc modification from the substrate proteins (25). In this regard, recent studies have revealed a role of histone H2B Ser-112 O-GlcNAc in the regulation of histone H2B Lys-120 ubiquitination and, consequently, in promoting transcriptional elongation (26, 27). The functional importance of OGT is manifested by its essential roles in ES cell viability and mouse embryonic development (28). Although OGT has been shown to interact with all three TET proteins and catalyze O-GlcNAcylation of TET1 and TET2, surprisingly, OGT was not found to significantly affect the activities of TET1/2/3 (19–21). Rather, the TET1/2/3 proteins were shown to play critical roles in targeting OGT to chromatin and enhancing OGT enzymatic activity (19–21). In this study, we show that, although OGT associates and catalyzes the O-GlcNAcylation of all three TET proteins, it differentially regulates their subcellular localization and enzymatic activity.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Antibodies**—For expression by transient transfection, the full-length cDNAs of human TET1, TET2, TET3, and OGT were constructed by ligating various fragments amplified by PCR from human H9 ES cells and then cloned into the pcDNA3.1/3×Myc-A or pcDNA3.1-FLAG vector to express Myc- or FLAG-tagged proteins. The human OGT used in our study corresponds to OGT isoform 3. The H508 of this isoform of OGT is equivalent to the H498 in the catalytic center of the OGT isoform 1 (29). Deletion mutants of OGT and TET3 were generated by PCR amplification and then cloned into pEGFP-C2 or pcDNA3.1-FLAG, respectively. The OGT (H508A) mutant in pcDNA3.1/3×Myc-A was created by PCR-directed mutagenesis. All plasmids were verified by DNA sequencing. The antibodies used were as follow: Myc (AbMART), GFP (AbMART), FLAG (Sigma-Aldrich), Actin (HuaAn Corp.), and O-GlcNAc (Abcam, catalog no. ab2739, mouse monoclonal RL2 antibody). The anti-5hmC antibody was a gift from Dr. Degui Chen (Shanghai Institute of Biochemistry and Molecular Biology (SIBCB), Chinese Academy of Sciences (CAS)).

**Cell Culture and Transient Transfection**—HeLa and HEK293T cells were routinely maintained with regular DMEM supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Transient transfection of HeLa and HEK293T cells was carried out using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer.

**Immunoadfinity Purification of FLAG-TET3-associated Proteins**—HEK293T cells were transfected with either the control vector or FLAG-TET3. Two days after transfection, the cells were harvested and washed with PBS twice. The cells were lysed with EBC buffer (20 mM Tris-HCl (pH 8.0), 125 mM NaCl, 2 mM EDTA, and 0.5% Nonidet P-40) with the addition of protease inhibitors. After removing cell debris by high-speed centrifugation, cell lysates were precleared with protein A-conjugated Sepharose beads for 2 h at 4 °C with gentle agitation. Then, the anti-FLAG M2 agarose beads were added and incubated for 3 h at 4 °C with rotation. Beads were washed five times with EBC buffer for 10 min each. SDS loading buffer was added directly to the beads and boiled for 5 min before samples were loaded and separated by 8% SDS-PAGE.

**Silver Staining**—Purified FLAG-TET3 and its associated proteins were loaded onto 8% SDS-PAGE for electrophoresis. The gel was fixed with 50% methanol at room temperature for 30 min, followed by further fixation with 5% methanol for 10 min. For staining, the gel was agitated in 50 ml of 32 μM DTT for 20 min, followed by incubation with 10⁻³ M AgNO₃ for 20 min. After washing with double-distilled H₂O, the gel was treated with 50 ml of image developer (3% Na₂CO₃, 25 μl 40% formaldehyde). The reaction was stopped using stop solution (12.5 ml acetic acid and 25g Tris in 500 ml of double-distilled H₂O) when clear bands were observed.

**Mass Spectrometry Analysis**—Protein identification by mass spectrometry was carried out in the Instrumental Analysis Center of Shanghai Jiaotong University. To determine the identities of the protein copurified with FLAG-TET3, the corresponding protein bands in the silver staining gel were excised and in-gel digested with trypsin. The tryptic peptide digests of the proteins were analyzed using an MDLC system (Michrom Bioreources Inc., Auburn, CA) coupled with a Thermo Finnigan two-dimensional linear ion trap mass spectrometer (LTQXL, Thermo Inc., San Jose, CA). The peptide sequences were determined by searching MS/MS spectra against the Human International Protein Index protein sequence database (IPI.Human.v3.63.fasta, 84,118 entries) by using the Turbo SEQUEST program in the BioWorks 3.3 software suite with a precursor ion mass tolerance of 2.0 Da and fragment ion mass tolerance of 1.0 Da.

**Immunofluorescence Staining of 5hmC**—For immunofluorescence staining of 5hmC, HeLa cells were washed with iced 1× PBS (137 mM NaCl, 2.7 mM KCl, 2 mM KH₂PO₄, and 10 mM Na₂HPO₄) prior to fixation in 4% fresh paraformaldehyde in PBS for 15 min. Then, the cells were treated with 2 M HCl at room temperature for 30 min, followed by neutralizing with 100 mM Tris-HCl (pH 8.0) at room temperature for 30 min and blocking with 5% BSA in PBS for 1 h in a 37 °C incubator. The incubation with 5hmC primary antibody was carried out at 4 °C overnight, and secondary antibody incubation was performed at room temperature for 1 h. DNA was stained with DAPI. Images were acquired using a Leica SP5 system.

**Immunoprecipitation**—For immunoprecipitation, HEK293T cells were lysed with lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM KCl, 1% Triton X-100, and 1 mM EDTA). The cell lysates were cleared by centrifugation at 14,000 rpm for 10 min at 4 °C. Supernatants were incubated with anti-FLAG M2 beads (Sigma) at 4 °C for 2 h. Beads were then washed four times with wash buffer (50 mM Tris-HCl (pH 7.5), 150 mM KCl, 0.1% Triton X-100, and 1 mM EDTA) and then boiled in 1× SDS loading buffer. The eluted precipitates were subjected to SDS-PAGE and Western blot analysis with the indicated antibodies.

**RESULTS**

**OGT Is a Major TET3-associated Protein**—Given the functional significance of TET proteins in the regulation of DNA
methylation and transcription, we wished to elucidate whether TET proteins function as protein complexes and how they are regulated. To this end, we expressed FLAG-tagged TET3 in 293T cells and carried out immunoaffinity purification of FLAG-TET3 and its associated proteins. A representative silver staining gel of purified FLAG-TET3 and its associated proteins is shown in Fig. 1A. In addition to the band corresponding to FLAG-TET3, a prominent band with a molecular mass of ~110 kDa that was copurified with FLAG-TET3 was subjected to mass spectrometry analysis. The majority of the identified peptide sequences matched with the sequence of human OGT (Fig. 1A). The interaction between TET3 and OGT was confirmed by coimmunoprecipitation (co-IP) of FLAG-TET3 and Myc-OGT expressed in 293T cells (Fig. 1B) as well as Myc-TET3 and Flag-OGT (Fig. 1C). All immunoprecipitations were performed with anti-FLAG antibody. Luc, luciferase.

**FIGURE 1.** OGT is a major TET3-interacting protein. A, silver staining gel showing the proteins copurified with FLAG-TET3 expressed in 293T cells. The Vector lane represents the proteins purified from 293T cells transfected with the control vector. The two major bands were excised and analyzed by mass spectrometry to be TET3 and OGT, respectively. The numbers and the positions of the identified OGT peptides are listed. B and C, reciprocal IP-WB analyses showing co-IP of coexpressed TET3 and OGT. Note that FLAG-TET3 and Myc-OGT were used in B and that FLAG-OGT and Myc-TET3 were used in C. All immunoprecipitations were performed with anti-FLAG antibody. Luc, luciferase. D, TET3 interacts with OGT through its C-terminal catalytic domain. Top panel, the organization of TET3 protein and the deletion mutants used for mapping the region required for OGT interaction. Various FLAG-tagged TET3 deletion mutants and the wild-type TET3 were coexpressed with GFP-OGT in 293T cells. Immunoprecipitations were performed with anti-FLAG antibody, and WB analyses were performed with either anti-GFP or anti-FLAG, as indicated. The results of OGT interaction are summarized in the top panel. E, the optimal interaction between OGT and TET3 requires both the N-terminal and C-terminal domains of OGT. Top panel, the organization of OGT protein and the deletion mutants used for analyzing the interaction with TET3. The experiments were performed essentially as in D. The results of TET3 interaction are summarized in the top panel. NLS, nuclear localization sequence.
FLAG-OGT expressed in 293T cells (Fig. 1C). As a negative control, luciferase was associated neither with TET3 nor OGT. Thus, in agreement with a recent publication (20), our data identified OGT as a major TET3-associated protein.

Mapping the Protein-Protein Interaction Domains of TET3 and OGT—Although TET3 has been reported to interact with OGT (19, 20), how these two proteins interact has not been investigated in detail. Thus, we wished to map the interaction domain(s) required for their interaction. We generated a series of FLAG-tagged TET3 deletion mutants (Fig. 1D, top panel) and analyzed their interaction with GFP-OGT, which was expressed better than Myc-tagged OGT, by co-IP assay. As shown in Fig. 1D, the OGT interaction region was mapped to the TET3 C-terminal dioxygenase domain (amino acids 1080–1660).

OGT comprises two distinct regions: an N-terminal region consisting of a series of tetratricopeptide repeats (TPR) and a C-terminal region containing the catalytic activity (Fig. 1E, top panel) (29). We generated a series of FLAG-tagged OGT deletion mutants (Fig. 1E, top panel) and examined their interaction with Myc-TET3 by co-IP. A representative result in Fig. 1E shows that, although IP of FLAG-OGT efficiently coprecipitated Myc-TET3, deletion of either the entire N-terminal TPR region or the first six TPR repeats substantially reduced the interaction of FLAG-OGT with Myc-TET3. Although FLAG-OGT with deletion of the C-terminal 146 amino acids (FLAG-OGTΔC) was expressed poorly, co-IP with Myc-TET3 could be observed at a reduced level in comparison with that of the full-length OGT. These results suggest that both the N-terminal and C-terminal regions of OGT are required for an optimal interaction with TET3. In agreement with this idea, the TPR region alone coprecipitated with Myc-TET3, but at a much reduced level compared with the full-length OGT (Fig. 1E). Therefore, we conclude that both the N-terminal TPR and the C-terminal catalytic domain of OGT are required for the efficient interaction of OGT with TET3.

OGT Catalyzes O-GlcNAc Modification of TET3—Although TET3 has been reported in previous studies (19, 20) to interact with OGT, it has not been shown whether OGT catalyzes TET3 O-GlcNAcylation. To address this issue, we coexpressed Myc-tagged OGT with a H508A mutation in the catalytic domain that is equivalent to H498A in the OGT isoform 1 (29), or an OGTΔC mutant with deletion of the C-terminal amino acids 901–1046 with FLAG-TET3 in 293T cells. The correct expression of these proteins was verified by WB analysis (Fig. 2A, Input). FLAG-TET3 was then immunoprecipitated using anti-FLAG antibody and examined for O-GlcNAc modification by WB analysis using an anti-O-GlcNAc antibody. The results in Fig. 2A showed that coexpression of the wild type but not the OGTΔC mutant resulted in substantial O-GlcNAcylation of TET3. Coexpression of the OGT H508A mutant resulted in a low level of TET3 O-GlcNAcylation in comparison with that of the wild-type OGT, suggesting that the H508A mutant, although substantially impaired, possesses a residual O-GlcNAc transferase activity.

To determine the O-GlcNAc modification site(s) of TET3, we coexpressed various FLAG-tagged TET3 deletion mutants with Myc-OGT. The TET3 deletion mutants were then immunoprecipitated with anti-FLAG antibody, and subsequent WB analysis using anti-O-GlcNAc antibody revealed O-GlcNAc of TET3 fragments of 1–680, 680–C, 680–1080, and 1080–C, but not the fragment of 1503–C (Fig. 2B), indicating that OGT is able to catalyze TET3 O-GlcNAcylation at multiple regions (sites).

OGT Affects TET3 Subcellular Localization in an O-GlcNAc Transferase Activity-dependent Manner—Having established that OGT interacts with and catalyzes O-GlcNAcylation of TET3, we next examined whether OGT regulates TET3 subcellular localization and/or enzymatic activity. Immunofluorescent staining revealed that, when expressed alone, Myc-TET3 exhibited a predominant nuclear staining pattern in HeLa cells (Fig. 2C). Consistent with previous studies (23, 30), OGT was found to be enriched in the nucleus but was also observed in the cytoplasm (Fig. 2C). Strikingly, Myc-TET3 exhibited a predominant cytoplasmic localization when it was coexpressed with FLAG-OGT. This drastic effect on TET3 subcellular localization was dependent on OGT enzymatic activity because Myc-TET3 maintained a predominant nuclear localization when it was coexpressed with the enzymatically defective FLAG-OGTΔC mutant or the H508A mutant with impaired enzymatic activity (Fig. 2C). This effect of OGT on TET3 subcellular localization was also observed in 293T cells and mouse NIH3T3 cells (Fig. 2D) and, thus, appears to be cell type-independent. We also noticed an increased cytoplasmic localization for FLAG-OGT when it was coexpressed with Myc-TET3 (Fig. 2C). We interpreted this result as a consequence of the interaction between FLAG-OGT and cytoplasmic Myc-TET3. Together, these results demonstrate for the first time that OGT regulates TET3 subcellular localization in a manner depending on the enzymatic activity of OGT.

TET3 Nuclear Localization Sequence—To understand how OGT regulates TET3 subcellular localization, we first investigated the determinant(s) of TET3 nuclear localization. Sequence analysis revealed a potential nuclear localization sequence, KKKR (from amino acids 1607 to 1610), in the C-terminal region of human TET3 (Fig. 3A, top panel). This sequence is critically important for TET3 nuclear localization because mutation of a single amino acid, arginine 1609 to alanine (R1609A), completely abrogated TET3 nuclear localization (Fig. 3A). In further support of the importance of this sequence in TET3 nuclear localization, we observed that the TET3(N1502) deletion mutant lacking the KKRK sequence is predominantly cytoplasmic, whereas the much shorter TET3(1503-C) mutant containing this sequence was predominantly nuclear (Fig. 3A).

OGT Promotes TET3 Nuclear Export—Because the TET3(1503-C) mutant resides in the nucleus as the full-length TET3, we analyzed whether OGT influenced its subcellular localization as it did on the full-length TET3. We found that coexpression of Myc-OGT with FLAG-TET3(1503-C) did not affect the nuclear localization of TET3(1503-C) (Fig. 3B). On the other hand, coexpression of Myc-OGT with FLAG-TET3(680-C) resulted in the cytoplasmic localization of TET3(680-C) (Fig. 3B). As controls, both FLAG-TET3(1503-C) and FLAG-TET3(680-C) were nuclear when they were expressed alone (Fig. 3B, bottom panel). The failure of Myc-OGT to affect FLAG-TET3(1503-C) nuclear localization is actually not due to the lack of interaction
between them because Myc-OGT was found to interact with both FLAG-TET3(1503-C) and FLAG-TET3(680-C), but not the negative control protein FLAG-Luc (Fig. 3C). Thus, OGT does not appear to drive OGT out of the nucleus by blocking the nuclear localization function of TET3. In support of this idea, addition of leptomycin B (LMB), a nuclear export inhibitor, was able to block FLAG-TET3 cytoplasmic localization induced by coexpressed Myc-OGT (Fig. 3D). Together, these results suggest that OGT is able to promote TET3 nuclear export through its ability to catalyze $O$-GlcNAc of TET3 within the region of amino acids 680–1502. However, because the TET3(1–680) can also be O-GlcNAcylated by OGT, we could not exclude the possibility that O-GlcNAcylation at the region of 1–680 could also affect TET3 subcellular localization.

**FIGURE 2.** OGT catalyzes TET3 O-GlcNAc modification and alters TET3 subcellular localization. A, OGT catalyzes TET3 O-GlcNAc modification in an enzymatic activity-dependent manner. FLAG-TET3 was expressed alone or together with wild-type Myc-OGT, the Myc-OGT(H508A) mutant, or the Myc-OGTΔC mutant in 293T cells. FLAG-TET3 was then immunoprecipitated from the corresponding cellular extracts and analyzed for O-GlcNAc modification by WB analysis. B, OGT catalyzes O-GlcNAc modification at multiple regions of TET3. FLAG-tagged TET3 and its various deletion mutants were coexpressed with Myc-OGT in 293T cells. The expression of various TET3 constructs was verified by WB analysis (left panel). Various TET3 proteins were immunoprecipitated using anti-FLAG antibody, and the O-GlcNAc modification was detected by WB analysis using an anti-O-GlcNAc antibody (right panel). C, OGT changes TET3 subcellular localization in an enzymatic activity-dependent manner. Myc-TET3 and FLAG-OGT were expressed either alone or together in HeLa cells, and immunofluorescence staining was performed accordingly. Note that Myc-TET3 was predominantly nuclear when expressed alone (left panel). However, Myc-TET3 was predominantly cytoplasmic when coexpressed with wild-type FLAG-OGT but not with the Myc-OGT(H508A) or Myc-OGTΔC mutants. D, OGT altered TET3 subcellular localization in 293T and NIH3T3 cells. The experiments were performed as in C.

OGT Negatively Regulates TET3 5hmC Activity, Most Likely through Its Effect on TET3 Subcellular Localization—Having established that OGT drives TET3 out of the nucleus, we investigated whether OGT also regulates TET3 enzymatic activity. By immunofluorescent staining using an anti-5hmC-specific antibody, we confirmed that expression of Myc-TET3 led to substantially increased levels of 5hmC in HeLa cells (Fig. 4, top row, cells marked with white arrows). However, when Myc-TET3 was cotransfected with FLAG-OGT and double immunofluorescent staining for Myc-TET3 and 5hmC were performed, we observed two types of Myc-TET3 subcellular localization: one with predominantly cytoplasmic Myc-TET3 (Fig. 4A, bottom row, cells marked with green arrows) and one with predominantly nuclear Myc-TET3 (Fig. 4A, bottom row, cells marked with white arrows). For cells with a nuclear Myc-TET3, a strong 5hmC staining was observed. However, for cells with a predominantly cytoplasmic Myc-TET3, 5hmC staining was substantially weaker and close to the levels in the untransfected HeLa cells (Fig. 4A). Given that coexpression of OGT resulted in TET3 cytoplasmic localization (Fig. 2, C and D), we...
interpreted the first type of cells as the cells expressing both Myc-TET3 and FLAG-OGT and the second type as the cells expressing Myc-TET3 but without, or with only a low level of, FLAG-OGT. Thus, OGT not only drives TET3 out of the nucleus but also down-regulates TET3 enzymatic activity.

To test whether OGT regulates TET3 activity through its effect on TET3 subcellular localization, we treated HeLa cells cotransfected with FLAG-OGT and Myc-TET3 with LMB to block TET3 nuclear export. TET3 5hmC activity was then examined by immunofluorescent staining. As expected, LMB treatment prevented Myc-TET3 cytoplasmic localization because all cells showed nuclearly accumulated Myc-TET3 (Fig. 4B). Notably, 5hmC staining revealed that each Myc-TET3-positive cell was also positive for 5hmC. Thus, blocking TET3 nuclear export driven by OGT is able to restore the 5hmC activity of TET3, suggesting that OGT down-regulates TET3 5hmC activity through its ability to regulate TET3 subcellular localization.

UDP-GlcNAc, the donor for O-GlcNAc modification, is derived from extracellular glucose through the cellular hexosamine biosynthesis pathway (25). To further test whether OGT regulates TET3 subcellular localization and 5hmC activity through O-GlcNAcylation, we treated HeLa cells transfected with Myc-TET3 and FLAG-OGT with 6-diazo-5-oxo-l-norleucine (DON), a specific hexosamine biosynthesis pathway inhibitor, and observed that DON treatment also blocked TET3 cytoplasmic localization (Fig. 4C). Consistent with the increased Myc-TET3 nuclear localization, DON treatment resulted in increased 5hmC staining in Myc-TET3-expressed cells (Fig. 4C, bottom row). WB analysis in Fig. 4D showed that DON treatment affected neither the expression of Myc-TET3 nor the expression of FLAG-OGT. Together, these results suggest that OGT regulates TET3 subcellular localization and, consequently, its 5hmC activity.

OGT Catalyzes O-GlcNAcylation of TET1 and TET2 but Affects Neither Their Nuclear Localization Nor Enzymatic Activity—Having established that OGT catalyzes TET3 O-GlcNAcylation and markedly alters its subcellular localization, we examined whether OGT also regulates TET1 and TET2 in a similar fashion. In the initial experiments, where full-length Myc-TET1 and FLAG-OGT were coexpressed in 293T cells, we could not consistently observe an interaction between these two proteins by co-IP assay, possibly because the full-length TET1 was not well expressed (data not shown).
found that the better expressed C-terminal region (amino acids 1418–2136) of TET1 (Myc-TET1C) that contains the 5hmC catalytic activity exhibited a weak interaction with FLAG-OGT in a co-IP assay (Fig. 5A). On the other hand, we found that the full-length TET2 was readily coprecipitated with FLAG-OGT in a co-IP assay (Fig. 5B). Thus, in parallel IP-WB experiments, OGT interacted with TET2 better than TET1, in agreement with two previous reports (19, 20) but not another one (21). We also found that OGT catalyzed O-GlcNAcylation of the full-length TET1 (Fig. 5C) and TET2 (Fig. 5D). Interestingly, despite the observed interaction and O-GlcNAcylation, OGT affected neither the nuclear localization of TET1 nor TET2 (Fig. 5, E and F). In addition, OGT did not affect the overall 5hmC enzymatic activity of all three TET proteins, it only affects TET3 subcellular localization and, consequently, the 5hmC enzymatic activity of TET3.

Glucose Concentration Regulates TET3 O-GlcNAcylation and Subcellular Localization—To test whether glucose metabolism influences TET3 O-GlcNAcylation and subcellular localization, we transfected HeLa cells with FLAG-TET3 and cultured the cells in medium with either a regular concentration of glucose (5 mM) or high glucose (25 mM) for 24 h. Subsequent WB analysis revealed that the culture in high-glucose medium resulted in increased TET3 O-GlcNAcylation (Fig. 6A), whereas immunofluorescent staining revealed an increased population of cells with cytoplasmic FLAG-TET3 (from ~11–37%) (Fig. 6, B and C). These results provide evidence that increased glucose concentration led to elevated TET3 O-GlcNAcylation and, consequently, increased TET3 nuclear export and cytoplasmic localization.

Because protein O-GlcNAcylation is controlled by the balancing activity of OGT and OGA (25), we also tested whether treatment with PUGNAc, an inhibitor of OGA (31), would affect TET3 O-GlcNAcylation as well as its subcellular localization. As shown in Fig. 6D, we first confirmed that treatment of HeLa cells with 150 μM PUGNAc led to a global increased protein O-GlcNAcylation. This treatment also notably increased the O-GlcNAcylation of transfected Myc-TET3 (Fig. 6E). Furthermore, this treatment also substantially increased the per-
The percentage of HeLa cells with cytoplasmically localized TET3 (Fig. 6F, from 9.8% in the control to 37.7% with PUGNAc treatment). These results demonstrate that TET3 O-GlcNAcylation is controlled by the balancing activities of endogenous OGT and OGA.

**DISCUSSION**

The finding that TET family proteins catalyze hydroxylation of 5mC to produce 5hmC has generated great interest regarding their roles in DNA-active demethylation and transcriptional regulation (6–8, 32). Functional studies have revealed the roles of TET proteins in various processes, including global and local DNA demethylation and ES cell pluripotency and differentiation, development, and oncogenesis. The functional importance of TET proteins also generated a great interest in purification and identification of TET-associated proteins. In addition to an earlier report on the association of TET1 with the Sin3A complex (18), recent studies from different laboratories all demonstrated the association of TET proteins with OGT (19–22). However, there are discrepancies among these studies in terms of how different TET proteins interact with OGT and whether they are O-GlcNAcylated by OGT. For example, in one study, TET1 was shown to be primarily required for targeting OGT to chromatin (21), whereas in other two studies, either TET2 or TET2 and TET3 were shown to interact more robustly with OGT and determine the chromatin association of OGT and the O-GlcNAcylation of histones (19, 20). In addition, although TET1 and TET2 were shown to be O-GlcNAcylated in one study (21), the O-GlcNAcylation of TET2 and TET3 was not detected in another study (20). In this study, we show that OGT is a major cellular protein copurified with TET3 (Fig. 1A). We showed that, although all three TET proteins interact with
OGT, TET1 exhibited a weaker interaction with OGT than TET2 and TET3 (Fig. 5), in agreement with the data from the last two studies (19, 20). On the other hand, we showed that all three TET proteins can be O-\text{GlcNAcylated} by OGT (Figs. 2 and 5). Our data are consistent with the study by Pasini and colleagues (21) showing O-\text{GlcNAcylation} of TET1 and TET2 and demonstrate, for the first time, the O-\text{GlcNAcylation} of TET3.

Although the previous studies all point to a protein-protein interaction between OGT and TET family proteins, a general conclusion is that OGT does not appear to regulate the expression and enzymatic activity of TET proteins (19–21). Rather, the TET proteins serve to bring OGT to chromatin to fulfill the function of histone O-\text{GlcNAcylation} (19–21). In this study, we show that OGT does not affect the subcellular localization and enzymatic activity of TET1 and TET2, data consistent with previous publications (19–21), OGT drives TET3 out of the nucleus (Fig. 2, C and D) and, consequently, inhibits TET3 enzymatic activity (Fig. 4). This effect on TET3 is dependent on OGT enzymatic activity because it was not observed for the enzymatic activity-defective OGT mutant (Fig. 2C). Thus, although TET proteins are required for targeting OGT to chromatin, OGT also uniquely regulates TET3 subcellular localization and enzymatic activity. This effect on TET3 is not cell type-specific because it is also observed in 293T and NIH3T3 cells (Fig. 2D). Because coexpression of OGT and TET3 not only results in TET3 cytoplasmic localization but also promotes OGT cytoplasmic localization (Figs. 2C and 3B), our data argue against a role of TET3 in targeting OGT to chromatin to regulate histone O-\text{GlcNAcylation} and H3K4 methylation (20).

Although the exact mechanism by which OGT promotes TET3 cytoplasmic localization is not known, OGT does not appear to directly block the nuclear localization function of TET3. We demonstrate that a single nuclear localization sequence, KKRK, determines the nuclear localization of human TET3 because a single amino acid mutation (R1609A) within this nuclear localization sequence is sufficient to abrogate TET3 nuclear localization (Fig. 3A). In support of our data, a recent study identified the conserved KKRK sequence as being required for nuclear localization of mouse TET3 (33). It is noteworthy that, although OGT appears to catalyze O-\text{GlcNAcylation} at multiple regions within TET3, it does not catalyze O-\text{GlcNAcylation} within the region of 1603-C that contains the nuclear localization sequence KKRK nor affect the nuclear localization of this region (Figs. 2B and 3B). OGT is able to catalyze the O-\text{GlcNAcylation} of TET3(680-C) and drives it out of the nucleus (Figs. 2B and 3B). Taken together, we favor a model postulating that OGT directly catalyzes O-\text{GlcNAcylation} of TET3 within the region of 680–
and that this modification somehow promotes TET3 nuclear export. Although a significant effort has been devoted to this study, we have yet to identify the exact serine or threonine residue(s) within this region whose O-GlcNAcylation results in cytoplasmic localization of TET3.

In agreement with the regulation of TET3 subcellular localization by OGT, we demonstrate that TET3 O-GlcNAcylation and subcellular localization are regulated by glucose concentration in the culture medium (Fig. 6, A–C) and also affected by treatment with DON (Fig. 4C) and PUGNAc (Fig. 6, E and F), the inhibitor for OGT and OGA, respectively. A high level of glucose led to increased O-GlcNAcylation of TET3 and increased TET3 cytoplasmic localization, presumably as a result of increased production of cellular UDP-GlcNAc, the donor for protein O-GlcNAcylation catalyzed by OGT. Thus, this finding thus reveals an unexpected link between glucose metabolism and DNA oxidation by TET3.

In conclusion, we demonstrate, in this study, that OGT differentially regulates the subcellular localization and enzymatic activity of TET family proteins. Although OGT interacts with and catalyzes the O-GlcNAcylation of all three TET proteins, it specifically promotes TET3 nuclear export and, consequently, inhibits the 5hmC activity of TET3. In addition, the cytoplasmically localized TET3 also promotes OGT3 cytoplasmic localization, presumably as a consequence of OGT-TET3 interaction.

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