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Recruitment of *O*-GlcNAc Transferase to Promoters by Corepressor mSin3A: Coupling Protein *O*-GlcNAcylation to Transcriptional Repression

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Summary

Transcription factors and RNA polymerase II can be modified by *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) monosaccharides at serine or threonine residues, yet the precise functional roles of this modification are largely unknown. Here, we show that *O*-GlcNAc transferase (OGT), the enzyme that catalyzes this post-translational modification, interacts with a histone deacetylase complex by binding to the corepressor mSin3A. Functionally, OGT and mSin3A cooperatively repress transcription in parallel with histone deacetylation. We propose that mSin3A targets OGT to promoters to inactivate transcription factors and RNA polymerase II by *O*-GlcNAc modification, which acts in concert with histone deacetylation to promote gene silencing in an efficient and specific manner.

Introduction

Gene transcription in eukaryotes is controlled elaborately in both spatial and temporal patterns by the dynamic interplay between transcriptional activation and repression. Genetic studies have revealed that either loss-of-function or gain-of-function mutations in proteins that are involved in transcriptional repression have severe impact on cell growth, differentiation, and apoptosis, thus underscoring an essential role of gene silencing in the biological functions of an organism (Ahringer, 2000; Burke and Baniahmad, 2000; Muller and Leutz, 2001). Multiple mechanisms are built into this process to ensure that a gene is turned off in an efficient and specific manner. Like transcriptional activation, repression must occur in the context of chromatin, where genomic DNA wraps around histone octamers to form nucleosomes. As a result, chromatin remodeling is one of the critical steps in gene silencing. Chromatin remodeling factors drive nucleosome mobilization by catalyzing ATP hydrolysis, and histone deacetylases (HDACs) remove acetyl groups from the histone tails. These two actions cooperatively alter the local chromatin conformation and create a repressive chromatin environment, limiting access of transcriptional activators and the general transcription apparatus to a promoter (Gregory et al., 2001; Kuzmichev and Reinberg, 2001; Wolffe et al., 2000).

Mammalian HDACs are divided into three classes: class I includes HDAC1, 2, 3, and 8 that are closely related to yeast Rpd3; class II comprises HDAC4–7, 9, and 10 that share homology with yeast Hda1 (Grozinger et al., 1999; Kao et al., 2002); class III are NAD^+-dependent histone deacetylases that resemble the yeast silencing protein Sir2 (Moazed, 2001; Vaziri et al., 2001). HDACs exist in diverse corepressor complexes that contribute to transcriptional silencing via distinct mechanisms (Burke and Baniahmad, 2000; Ng and Bird, 2000). One such corepressor complex is the Sin3-HDAC complex, in which Sin3 appears to act as a scaffold for the assembly of HDAC1, HDAC2, RbAp46, RbAp48, SAP18, and SAP30 subunits (Hassig et al., 1997; Zhang et al., 1997). The Sin3-HDAC complex can be recruited to target promoters by the direct or indirect association with an array of DNA binding repressors (Glass and Rosenfeld, 2000; Kouzarides, 1999). Mammalian Sin3 (mSin3) includes two isoforms, mSin3A and mSin3B, which are homologous to yeast corepressor Sin3 (Ayer et al., 1995). mSin3 can bind directly to transcription factors involved in a broad range of cellular functions (Burke and Baniahmad, 2000 for review). Moreover, mSin3 can contact unliganded nuclear hormone receptors indirectly through its interaction with the corepressor SMRT or N-CoR, so as to target HDAC activity to these repressors (Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997; Kao et al., 2000). In addition to mSin3, the other components of the mSin3-HDAC complex can serve as the targets of specific transcriptional repressors. HDAC1 and HDAC2 interact directly with YY1 and Rb, respectively (Brehm et al., 1998; Luo et al., 1998; Yang et al., 1996), whereas SAP30 is likely to couple the corepressor complex to a subset of nuclear hormone receptors (Laherty et al., 1998; Zhang et al., 1997).

Interestingly, the methyl-CpG binding protein MeCP2 can recruit the Sin3-HDAC complex to CpG-methylated DNA by binding to mSin3A, suggesting that histone deacetylation plays an important role in methylated gene silencing too (Jones et al., 1998; Nan et al., 1998; Ng et al., 1999). Several DNA methyltransferases, which are responsible for the generation and/or maintenance of gene methylation patterns, can also recruit HDAC activity for synergistic gene silencing (Fuks et al., 2001 for review). Also, a subset of the components of the mSin3-HDAC complex, such as mSin3A, HDAC2, and RbAp48, can be detected in human chromatin remodeling complexes (SWI/SNF; Sif et al., 2001), suggesting a direct link between histone deacetylation and chromatin remodeling in the repression of transcription. Together, these studies imply the general importance of histone deacetylation in gene silencing.

mSin3A and its homologs contain four putative paired amphipathic helix (PAH) domains, which are important for protein-protein interactions (Ayer et al., 1995). The linker region between PAH3 and -4 mediates mSin3A association with HDACs (Laherty et al., 1997). Although it has been established that Sin3 functions via HDACs, there is evidence that transcriptional repression by Sin3 also occurs independently of deacetylation. That is, mutants of mSin3 that do not interact with HDACs retain partial ability to repress transcription (Laherty et al.,

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1997; Wong and Privalsky, 1998). Furthermore, the abrogation of the catalytic activity of HDACs by mutagenesis or inhibitors does not completely impede the function of Sin3 in transcriptional silencing (Hassig et al., 1997; Kadosh and Struhl, 1998; Laherty et al., 1997; Nagy et al., 1997; Wong and Privalsky, 1998). Nevertheless, how Sin3 mediates HDAC-independent transcriptional repression is largely unclear.

Many nuclear and cytoplasmic proteins are dynamically modified by the *O*-linkage of the monosaccharide, *N*-acetylglucosamine (*O*-GlcNAc), to serine or threonine residues (Wells et al., 2001). This modification is catalyzed by *O*-GlcNAc transferase (OGT), an enzyme that is essential for cell survival (Shafi et al., 2000). The amino terminus of OGT contains multiple tandem tetratricopeptide repeats (TPR). The TPR is composed of a 34-amino acid motif that forms amphipathic α helices to mediate protein-protein interactions (Kreppel et al., 1997; Lubas et al., 1997; Shafi et al., 2000). The TPR domain is required for OGT multimerization, as well as for optimal recognition of protein substrates (Kreppel and Hart, 1999; Lubas and Hanover, 2000). The catalytic activity of OGT resides in the C terminus. RNA polymerase II (RNAPII) and a growing body of transcription factors can be modified by *O*-GlcNAc. Our previous studies have shown that *O*-GlcNAc modification of an Sp1 activation domain inhibits its protein-protein interactions and transcriptional potency, thereby providing direct evidence that *O*-GlcNAc can block the activity of a transcriptional activator (Roos et al., 1997; Yang et al., 2001). In addition, the carboxy-terminal domain (CTD) of the largest subunit of RNAPII is dynamically modified by multiple *O*-GlcNAc moieties that might act to arrest transcriptional elongation (Kelly et al., 1993). These findings raise the possibility that OGT might be involved in transcriptional repression. In the present study, we demonstrate that mSin3A can recruit OGT to the promoters of genes to repress transcription cooperatively, indicating that the interaction between mSin3A and OGT represents a mode of HDAC-independent repression by mSin3A.

Results

Tethering OGT to Promoters Enhances Transcriptional Repression

The observation that overexpression of OGT inhibited Sp1-driven transcription suggested that OGT plays a role in transcriptional repression (Yang et al., 2001). Then, does OGT modulate the activities of transcription factors independent of DNA or must it be targeted to the promoter region to exert its functions in a gene-specific manner? As a first step, we fused OGT to the Gal4 DNA binding domain (Gal4 DBD) so that the OGT could be artificially tethered to the upstream-activating sequence (UAS) within a promoter driving a luciferase reporter gene (Figure 1C). Transient expression of either OGT or Gal4-OGT inhibited basal transcription driven by the minimal promoter in a dose-dependent pattern. However, Gal4-OGT was more potent at transcriptional repression than OGT alone. This observation suggested that the recruitment of OGT to the gene promoter enhanced its inhibitory effect on transcription (Figure 1A). To test whether Gal4-OGT affected Sp1-activated transcription, six tandem GC boxes were introduced into the promoter either upstream or downstream of the UAS sequence (Figure 1C). Expression of low levels of OGT was ineffective in repressing both basal and Sp1-activated transcription. In contrast, the Gal4-OGT chimera markedly reduced Sp1-activated transcription, regardless of the position of GC boxes relative to UAS (Figure 1B). However, Gal4-OGT inhibited Sp1-activated transcription to a lesser extent than it inhibited basal transcription, indicating that the action of Sp1 may partially overcome the inhibitory effect of OGT (Figure 1B).

We then mapped the regions within OGT responsible for transcriptional repression. OGT (1–485) containing all 11 TPR motifs but lacking the C-terminal catalytic domain, retained considerable ability to repress basal transcription when recruited to the G_5-Luc promoter by the Gal4 DBD (Figure 1D). However, this truncated form was less able to repress transcription than full-length OGT, demonstrating that both the N-terminal TPR domain and the C-terminal catalytic domain are required for full repression by OGT. Analysis of additional deletion mutants showed that OGT (1–286) that spans the first six TPR motifs was sufficient to inhibit basal transcription. However, further N-terminal deletion indicated that TPRs 2–6 were inactive as a repressor. To determine whether the first six TPR motifs were also necessary for OGT-mediated inhibition, the TPR motifs 1–6 were deleted from full-length OGT. The activity of this mutant was significantly reduced. Inhibition of Sp1-activated transcription by the various OGT mutants resembled the observations on basal transcription, except that inhibition was to a lesser extent (Figure 1E). These results indicate that the TPR motifs 1–6 of OGT possess significant repressive activity that is separable from repressive activity of the catalytic domain.

OGT Interacts with mSin3A In Vitro

It was unexpected to observe that the N-terminal six TPR motifs of OGT were sufficient to mediate transcriptional repression. At least two distinct models could explain this observation. First, the TPR domain is required for OGT multimerization (Kreppel and Hart, 1999), thus OGT (1–286) fused to Gal4 DBD may recruit endogenous and catalytically active OGT to the target promoter. The second model is that Gal4-OGT (1–286) may recruit a transcriptional corepressor that brings about repression.

To test the first model, we mapped the precise region of OGT that mediates homomultimerization using a GST pull-down assay. The results showed that the OGT fragment corresponding to the TPR motifs 2–6 was fully capable of binding to [^{35}S]-full-length OGT (Figure 2A, compare lane 8 with lanes 3 and 4). Moreover, the TPR motifs 3–6 or 4–6 partially retained the ability to bind to full-length OGT (Figure 2A, lanes 9 and 10). However, amino acids 1–248, 1–214, and 1–180 of OGT that encompass the TPR motifs 1–5, 1–4, and 1–3, respectively, showed very weak interactions with full-length OGT (Figure 2A, lanes 5–7). Therefore, in addition to number of the tandem TPR motifs, the relative position or sequence specificity of the TPR motifs within the N-terminal TPR domain accounts for the strength of the protein-protein interaction. Hence, distinct regions in the TPR cluster of

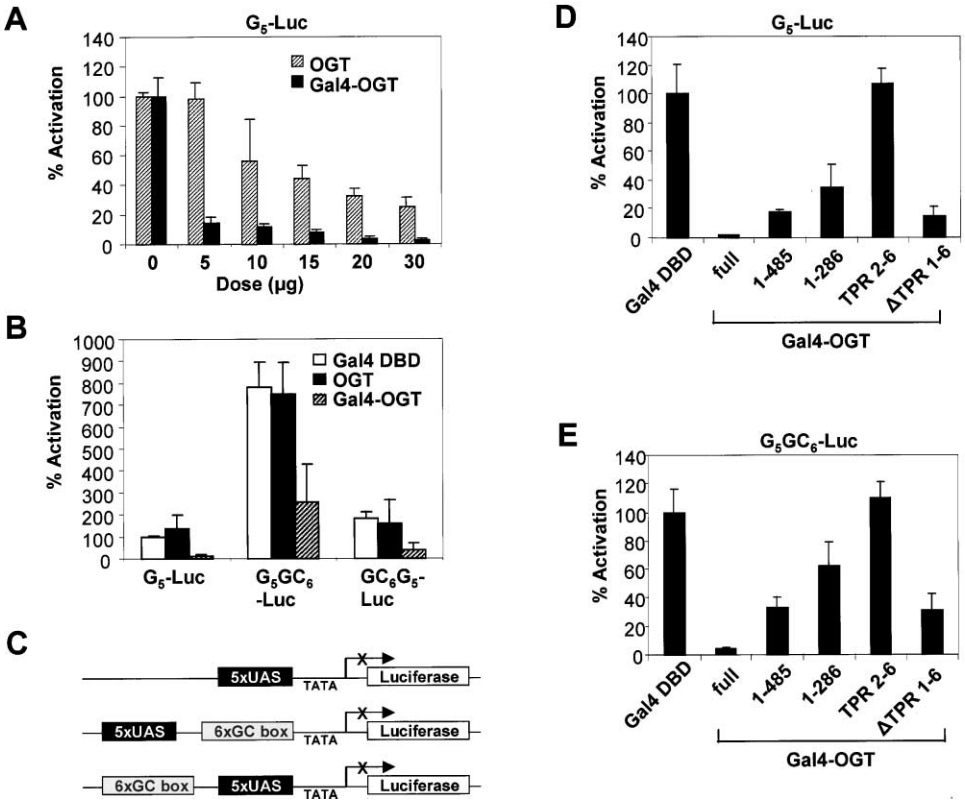

Figure 1. Tethering OGT to Promoters Potentiates its Transcriptional Repression

(A and B) Dose-response of OGT alone and Gal4-OGT in repressing basal (A) or Sp1-driven (B) transcription. Increasing amounts of an expression vector for Gal4-OGT or OGT alone were transiently cotransfected into $HepG_2$ cells with 20 μg of the reporter construct as indicated.

(C) Schematic diagram of reporter constructs used in transient transfection assays. Basal transcription was monitored with the G_5-Luc reporter construct containing five tandem UAS elements upstream of a minimal adenovirus major later promoter fused to the luciferase gene; Sp1-driven transcription was monitored with the G_5GC_6-Luc and GC_6G_5-Luc reporters in which six tandem GC boxes are placed downstream or upstream of the UAS elements in the G_5-Luc reporter, respectively.

(D and E) Mapping the regions within OGT involved in repression. Vectors expressing a series of deletion mutants of OGT fused to Gal4 DBD were transfected into $HepG_2$ cells with G_5-Luc (D) or G_5GC_6-Luc (E) reporters.

OGT may exhibit specific conformations. This property may contribute to the ability of OGT to *O*-GlcNAcylate a diverse range of protein substrates with no discernable motif. Since OGT (TPR 2–6) is sufficient to bind full-length OGT yet this OGT fragment fails to repress both basal and Sp1-activated transcription (Figures 1D and 1E), then recruitment of endogenous OGT by Gal4-OGT deletion derivatives is insufficient for transcriptional repression.

To explore the second model that Gal4-OGT (1–286), containing TPR motifs 1–6, may recruit a transcriptional corepressor, we chose mSin3A as a candidate. The corepressor mSin3A contains four putative paired amphipathic helix (PAH) domains (Ayer et al., 1995). Since the TPR motif also forms amphipathic α helices, the potential for hydrophobic interaction between OGT and mSin3A was investigated.

To determine whether OGT can associate directly with mSin3A, immobilized GST-OGT was incubated with [^{35}S]-mSin3A. As shown in Figure 2C, GST-OGT pulled down mSin3A specifically (top, compare lane 4 with lane 3), indicating that these proteins can physically interact directly in vitro. Analysis of the set of N- and C-terminal deletion mutants of OGT showed that TPR motifs 1-6 were the minimal region sufficient for this mSin3A interaction (Figure 2C, top and bottom). Removal of this region from full-length OGT abolished the interaction with mSin3A, demonstrating that the TPR (1–6) region was also necessary for OGT binding to mSin3A (Figure 2C, lane 8 in bottom).

To find the region in mSin3A that interacts with OGT, various [^{35}S]-mSin3A fragments fused to the Gal4 activation domain (AD) were incubated with immobilized GST-OGT (1–286). Amino acids 888–967 of mSin3A, corresponding to the PAH4 domain, were sufficient to mediate interaction with the TPR motifs 1–6 of OGT (Figure 2E, lane 7). In contrast, the fragments containing PAH 1–2 (amino acids 1–386) or PAH 2–3 (amino acids 302–529) did not display significant interaction with OGT (Figure 2E, lanes 3 and 4).

In summary, the TPR motifs 1–6 are sufficient and necessary for OGT interaction with the corepressor mSin3A at the PAH4 domain. Removal of the first TPR motif obliterated this physical interaction with mSin3A, as well as the ability of the TPR cluster to repress transcription (Figures 1D and 1E), but this deletion did not abolish the interaction with full-length OGT. These observations strongly support the second model in which

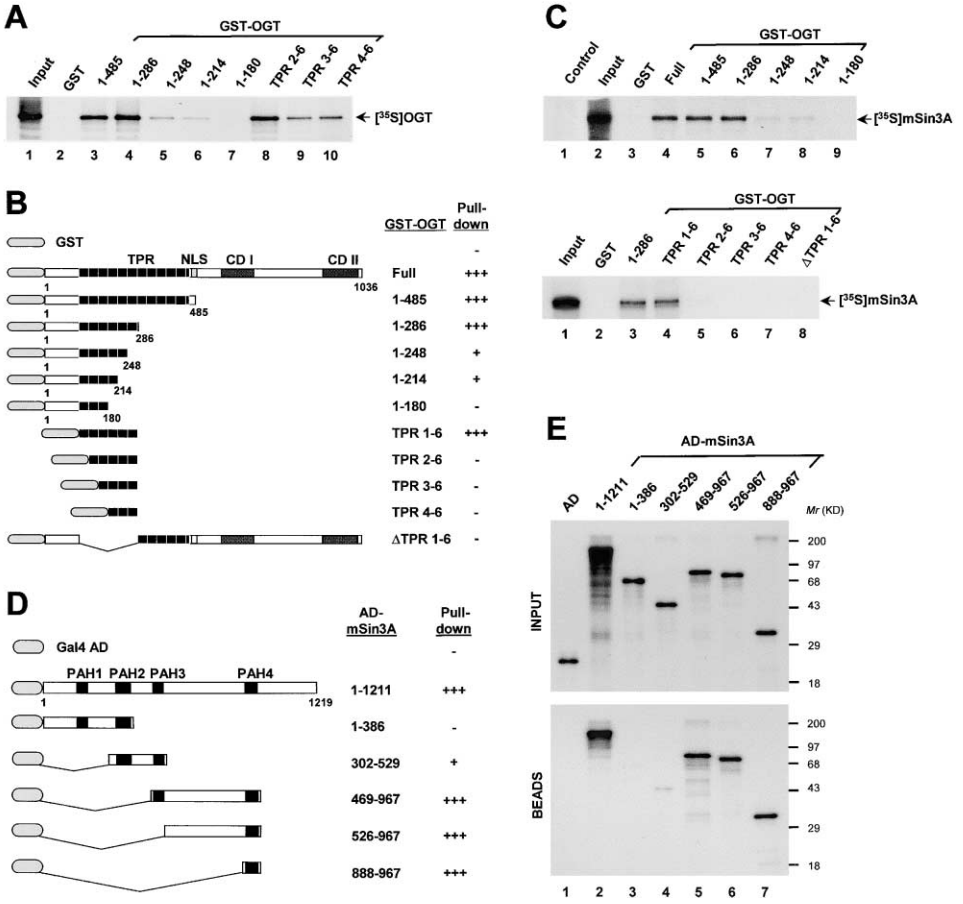

Figure 2. OGT Interacts with mSin3A In Vitro

(A) TPR motifs 2–6 mediate OGT self-association. [35S]-OGT was synthesized in reticulocyte lysates and incubated with equal amounts of various deletion mutants of OGT fused with immobilized GST. Input represents 30% [35S]-OGT.

(B) Schematic representation of full-length OGT and various deletion mutants. The affinity of the OGT mutants for [35S]-mSin3A in GST pull-down experiments is depicted (+++, strong; +, weak; -, no interaction).

(C) TPR motifs 1–6 in OGT interact with mSin3A. [35S]-mSin3A was incubated with the indicated GST-OGT deletion mutants; unprogrammed reticulocyte lysate was the negative control (top, lane 1). The top shows binding of mSin3A to C-terminal truncated OGT; the bottom shows the binding to the N-terminal deletion mutants. Input represents 20% of [35S]-mSin3A.

(D) Schematic representation of mSin3A deletion mutants and their affinity for OGT(1–286).

(E) mSin3A PAH4 domain interacts with OGT. [35S]-mSin3A deletion mutants fused to Gal4 AD were incubated with immobilized GST-OGT (1–286) and then analyzed by fluorography. The input (20%) and bound fractions are shown in the respective panels.

the OGT TPR domain mediates repression by recruiting the corepressor mSin3A.

OGT Interacts with mSin3A In Vivo

To ascertain whether the physical interactions between OGT and mSin3A that we observed in vitro are reflected by interactions in vivo, a mammalian two-hybrid assay was conducted. When mSin3A PAH4 domain (amino acids 888–967) fused to Gal4 AD was coexpressed with OGT (1–286) fused to Gal4 DBD in $HepG_2$ cells, the interaction between the two fusion proteins resulted in a marked increase in luciferase gene transcription from the UAS-containing promoter (Figure 3A). As a control, coexpression of the Gal4 AD-mSin3A (1–192) fusion that includes the PAH1 domain with Gal4 DBD-OGT (1–286) fusion failed to induce luciferase expression. This result confirmed that a specific interaction between the OGT (TPR 1–6) region and the mSin3A PAH4 domain can occur in vivo.

We next conducted coimmunoprecipitation experiments to determine whether OGT and mSin3A form a complex in living cells. After cotransfection of Cos-7 cells with the expression vectors for mSin3A and HA epitope-tagged OGT, HA-OGT was immunoprecipitated from cell lysates using α-HA antibody (Figure 3B). Immunoblotting analysis showed that both exogenous mSin3A and a detectable amount of endogenous mSin3A and HDAC1 were coprecipitated with OGT even from the cells transfected with the HA-OGT expression vector only (Figure 3B). These data indicated that OGT and mSin3A form a complex in vivo. The reprobed blot indicated the presence of HDAC1 in the OGT-mSin3A complex, but HDAC4 and Sp1 were not (Figure 3B). Of note, a small fraction of mSin3A and HDAC1 were associated with OGT (Figure 3B, compare lane 6 with lane 3), sug-

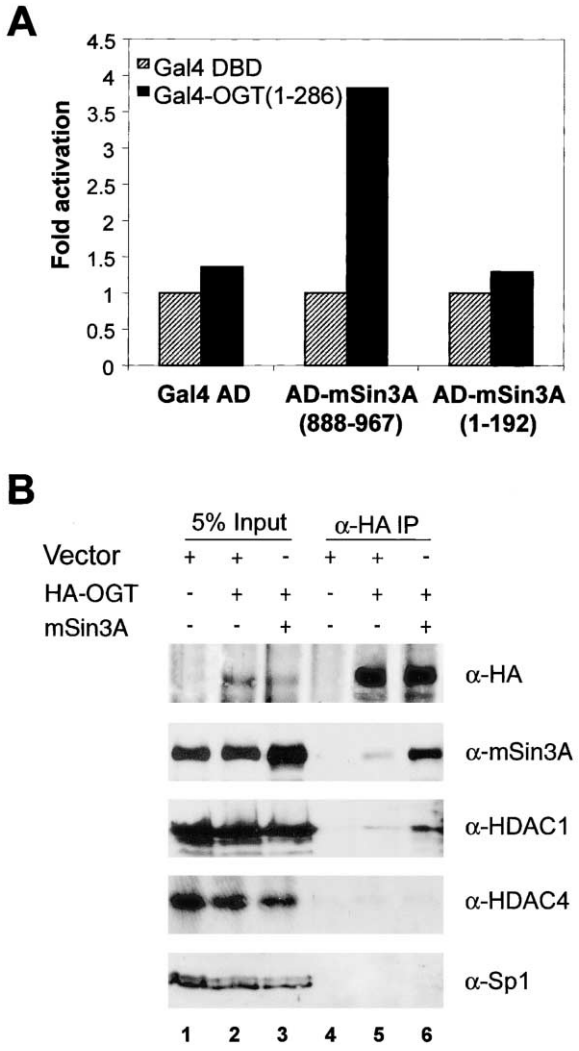

Figure 3. OGT Interacts with mSin3A In Vivo

(A) Mammalian two-hybrid analysis. Vectors expressing Gal4 AD, AD-mSin3A PAH4, and AD-mSin3A PAH1 domains were cotransfected individually into $HepG_2$ cells with vectors expressing either OGT (1–286) fused to Gal4 DBD or Gal4 DBD alone, together with the G_5-Luc reporter. The results are expressed as a fold activation obtained through the interaction of mSin3A deletions with OGT (1–286) over the baseline reporter activity generated by the interaction between the deletion mutants and Gal4 DBD alone.

(B) Coimmunoprecipitation analysis. Cos-7 cells were transfected with HA-OGT expression vector in the absence or presence of mSin3A expression vector. HA-OGT was immunoprecipitated with α-HA antibody. The precipitates were immunoblotted with various antibodies as indicated. Input represents 5% of whole-cell lysates used for immunoprecipitation.

gesting that this complex is in the minority among the diverse corepressor complexes involving mSin3A and/or HDAC1. Together, these observations suggest a physical and functional relationship between OGT, mSin3A, and HDAC1.

OGT and mSin3A Cooperatively Repress Transcription

Thus far, we have shown that artificially directing OGT to promoters with a Gal4 DBD potentiates OGT-mediated repression. Since mSin3A is recruited to promoters by sequence-specific DNA binding repressors to repress transcription and since there is a physical interaction between OGT and mSin3A, we propose that OGT could be recruited in nature to promoters by mSin3A to exert its inhibitory effect on transcription.

To test this idea, we examined the effects of mSin3A fused to Gal4 DBD (Gal4-mSin3A) and OGT on the activity of the Gal4-dependent reporters in transient transfection assays. When low doses of Gal4-mSin3A and OGT were expressed in $HepG_2$ cells alone, neither inhibited either basal or Sp1-activated transcription. However, coexpression of low doses of Gal4-mSin3A and OGT significantly reduced transcription (Figures 4A and 4C). These results revealed that OGT and mSin3A synergistically repressed basal and Sp1-activated transcription. Deletion of either TPR 1–6 in OGT or the PAH4 region in Gal4-mSin3A abrogated the synergism between the two proteins, indicating that mSin3A indeed recruited OGT via the mapped domains (PAH4 of mSin3A with TPR 1–6 of OGT) to the promoters for their functional cooperation in repression (Figures 4A and 4C). As shown in Figures 4B and 4D, high-level expression of Gal4-mSin3A or its ΔPAH4 mutant alone dramatically decreased transcription, presumably because of the OGT-independent mechanisms of mSin3A repression that dominate at the high dose. As a result, synergistic action between OGT and Gal4-mSin3A could not be easily observed at this high dose. These findings illuminate a functional interaction between OGT and mSin3A at the promoter region of a target gene.

OGT and HDACs Can Function in Parallel Pathways

In addition to targeting histone deacetylase activity to promoters, Sin3 also appears to repress transcription in an HDAC-independent manner (Hassig et al., 1997; Kadosh and Struhl, 1998; Laherty et al., 1997; Nagy et al., 1997; Wong and Privalsky, 1998). However, the molecular mechanisms underlying this HDAC-independent repression have not been defined. Given that a protein complex containing OGT, mSin3A, and HDAC1 is present in cells, two models can be proposed that address the relationships among the three components. First, mSin3A might direct OGT and HDAC1 into parallel and independent pathways to silence gene transcription; second, mSin3A might serve as a platform to colocalize OGT and HDAC1. This colocalization could allow OGT to modify and activate HDAC1. This second model would predict that OGT functions via its catalytic activity to repress gene transcription in an HDAC-dependent pathway.

The PAH4 region in mSin3A is both sufficient (Figures 2E and 3A) and necessary (Figure 5A) for binding OGT. The linker region between PAH3 and PAH4 mediates the mSin3A association with HDACs (Laherty et al., 1997; Wong and Privalsky, 1998). Thus, Gal4-mSin3A (ΔPAH4) should interact with HDAC1 but not OGT. As shown above (Figure 4D), high-level expression of both Gal4-mSin3A and the ΔPAH4 mutant substantially and similarly inhibited the transcriptional activity of the Gal4-dependent reporter (Figure 5B). However, the behaviors of Gal4-mSin3A and the ΔPAH4 mutant were distin-

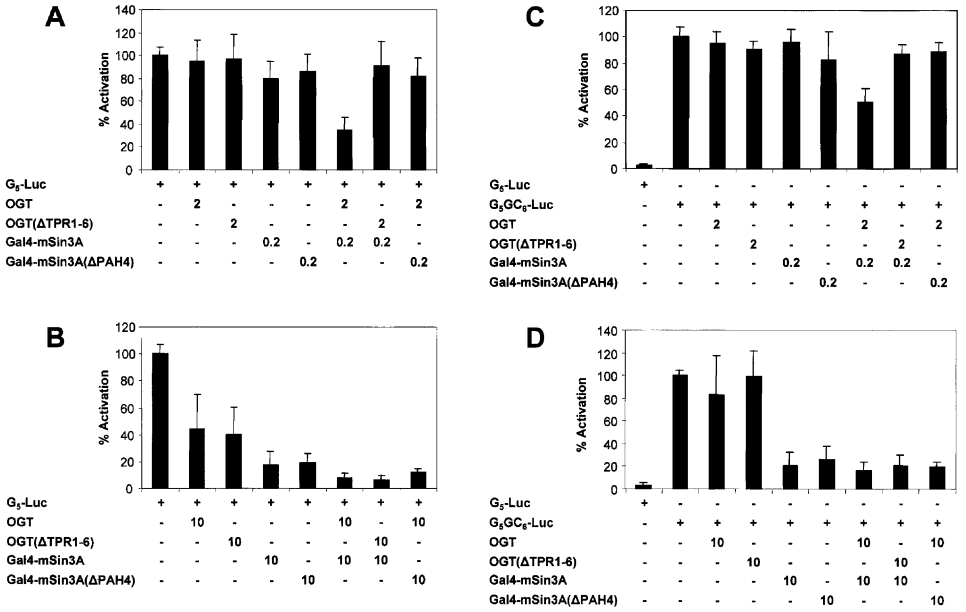

Figure 4. OGT and mSin3A Cooperatively Repress Transcription

OGT and mSin3A repress basal (A and B) and Sp1-driven (C and D) transcription in synergy. $HepG_2$ cells were transiently cotransfected with expression vectors for OGT, OGT (ΔTPR 1–6), Gal4-mSin3A, and Gal4-mSin3A (ΔPAH4) either individually or in combination, together with either the G_5-Luc (A and B) or G_5GC_6-Luc (C and D) reporter construct (20 μg). The amounts of expression vectors in micrograms are indicated in each panel. The luciferase activity was normalized to β-galactosidase activity and is presented as a percentage of the values obtained from transfection with the empty expression vector.

guishable in the presence of an HDAC inhibitor, trichostatin A (TSA). While wild-type Gal4-mSin3A retained considerable repressive activity despite the blockade of the mSin3A-HDAC pathway with increasing doses of TSA, the ΔPAH4 mutant, that abolished mSin3A association with OGT, had a reduced repressive activity in the presence of the HDAC inhibitor (Figure 5B). This result supports the earlier findings that mSin3A repression has an HDAC-independent component. Part of this HDAC-independent component depends on the PAH4 domain, the domain that binds OGT. This result suggests that OGT can act independently of HDACs to repress transcription. TSA treatment also increased basal transcription from the reporter in the presence of Gal4 DBD, suggesting the reporter is partially repressed by means of packaging the reporter plasmid into nucleosomes in cells (Figure 5B). However, even at a maximally effective dose of TSA, transcription was only restored to about 50% with the PAH4 deletion mutant as compared to the Gal4 DBD alone. Hence, this experiment does not exclude additional mechanisms that may underlie mSin3A-induced HDAC-independent transcriptional repression. In support of this notion, there is evidence that mSin3A PAH3 domain binds to the general transcription factor TFIIB and could interfere with its function (Wong and Privalsky, 1998). Nevertheless, genetic studies are expected to shed new light on the functional relationships among OGT, mSin3A, and HDACs.

Both the TPR and the C-terminal catalytic domain of OGT appear to participate in gene repression (Figure 1D). To determine the independent role of the catalytic domain, we made use of the report that deletion of a short C-terminal segment (amino acids 945–1036) renders OGT catalytically dead (Lubas and Hanover, 2000). When cells were transfected with a low dose (1 μg) of the vector expressing this catalytically dead mutant (Gal4-OGT [Δcat]), this fusion protein failed to repress transcription from the G_5-Luc reporter (Figure 5D). In contrast, low-level expression of Gal4-OGT(full-length), the TPR 1–6 deletion mutant (Gal4-OGT [ΔTPR 1–6]), and Gal4-OGT (471–1036) that encompasses the catalytic domain but lacks the entire TPR domain (Figures 5D and 5E) each repressed transcription 2-fold. These data strongly suggest that the catalytic activity of OGT is required for repression and it plays a predominant role at a low protein level when the enzyme is artificially targeted to the promoter. At higher levels of protein expression, which would promote the recruitment of the rest of the repression complex, Gal4-OGT (ΔTPR 1–6) exhibited less repressive activity than Gal4-OGT (Figure 5D). Introduction of the catalytically dead mutation into the TPR 1–6 deletion mutant (Gal4-OGT [ΔTPR1–6 Δcat]) further abrogated repressive activity of OGT, revealing that TPR 1–6 and the catalytic function of OGT additively contribute to transcriptional inhibition (Figure 5D). Of note, a high dose of Gal4-OGT (ΔTPR1–6 Δcat) retained residual repressive activity (Figure 5D), perhaps conferred by a region within TPR 7–11 (Figure 5E) where we have detected the interaction of with other corepressors (X.Y. and J.E.K., unpublished data). Thus, OGT, when tethered to a promoter through the Gal4 DBD, exerts at least two separable repressive effects on reporter gene expression: one is attributable to its catalytic activity, another to the recruitment of endogenous mSin3A-HDAC complex via its TPR 1–6. Of note, these two effects are manifest at different protein expression levels.

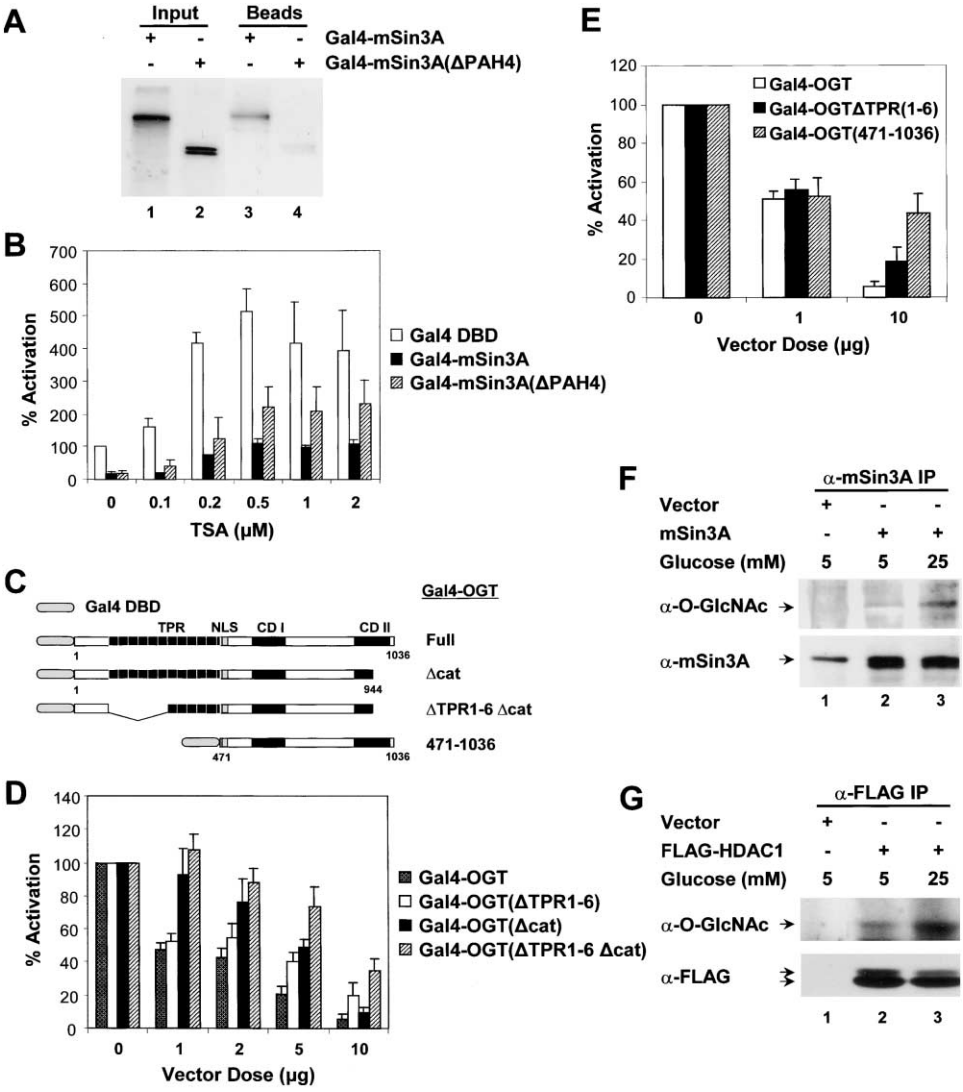

Figure 5. OGT and HDACs Can Function in Parallel Pathways

(A) PAH4 domain is required for Gal4-mSin3A interaction with OGT. [^{35}S]-Gal4-mSin3A and [^{35}S]-Gal4-mSin3A (ΔPAH4) were incubated with immobilized GST-OGT. Input represents 20% of the radiolabel. Gal4 DBD did not bind to GST-OGT (data not shown).

(B) Deletion of the PAH4 domain renders mSin3A less potent at transcriptional repression in response to TSA. HepG$_2$ cells were cotransfected with the G$_5$-Luc reporter and an expression vector for Gal4 DBD alone or fused to mSin3A or its PAH4 deletion mutant. 24 hr posttransfection, cells were treated with indicated doses of TSA and incubated for an additional 18 hr, followed by the luciferase assay.

(C) Schematic representation of full-length OGT and various deletion mutants in fusion with Gal4 DBD.

(D and E) Additive effect of the TPR 1–6 region and the catalytic domain of OGT on transcriptional repression. HepG$_2$ cells were transfected with G$_5$-Luc reporter (20 μg) and the indicated amounts of the expression vector for Gal4-OGT or its deletion mutants. Total DNA was equalized by the addition of an expression vector for Gal4 DBD alone. Results in each transfection were normalized to the activity of the reporter in the presence of 10 μg of the Gal4 DBD expression vector at a value of 100%.

(F and G) mSin3A and HDAC1 are *O*-GlcNAcylated proteins. mSin3A (F) or FLAG-HDAC1 (G) were overexpressed in Cos-7 cells grown in normal glucose (5 mM) or high glucose (25 mM) medium, immunoprecipitated with α-mSin3A or α-FLAG antibody, and then analyzed by immunoblot with α-*O*-GlcNAc and α-mSin3Aor α-FLAG antibodies. Cells transfected with an empty expression vector served as a control.

At low levels, the catalytic activity of OGT dominates because it is brought to the promoter by its covalent linkage to the Gal4 DBD. At high levels, the noncovalent interactions with corepressor complexes are sufficient for full repression.

Given that OGT catalytic activity is, at least in part, responsible for HDAC-independent repression, what are the downstream targets of OGT? Recently, we showed that overexpression of OGT inhibited the Sp1-driven transcription from a heterologous promoter (Yang et al., 2001). Here, we show that overexpression of OGT also inhibits basal transcription from a minimal promoter (Figure 1A). These observations hint that Sp1 and components of basal transcription machinery such as the CTD of RNAPII could serve as specific targets of OGT.

Because OGT comes into close association in the repressive complex with mSin3A and HDAC1, the enzyme might modify these proteins. To determine if they

are modified, mSin3A and FLAG epitope-tagged HDAC1 were transiently expressed in Cos-7 cells and were immunoprecipitated using anti-mSin3A and anti-FLAG antibodies, respectively. Subsequent immunoblotting analysis using an α-*O*-GlcNAc antibody indicated that mSin3A and HDAC1 were modified by *O*-GlcNAc (Figures 5F and 5G) and that the stoichiometry of modification increased in high-glucose (25 mM) medium (Figures 5F and 5G). That both of these proteins are *O*-GlcNAcylated is further evidence that OGT interacts with them catalytically. Because the functional significance of *O*-GlcNAcylation of mSin3A and HDAC1 has yet to be determined, we cannot exclude the possibility that OGT can also modulate HDAC1 activity, thereby mediating transcriptional repression via both HDAC-dependent and -independent pathways (Figure 7).

Proteins at Silenced Promoters Are Hyperglycosylated

The above experiments identified a physical and functional interaction between OGT and mSin3A. To determine if this interaction has physiological relevance on endogenous genes, a chromatin immunoprecipitation (ChIP) assay was performed. It is known that an unliganded estrogen receptor nucleates a transcriptional repression complex containing mSin3A-HDAC corepressors on estrogen-responsive genes. Estrogen binding triggers the release of the corepressors and the subsequent recruitment of histone acetyltransferase coactivators for gene activation (Chen et al., 1999; Shang et al., 2000). In the ChIP experiment, proteins bound to the estrogen target genes (pS2, EB1, and cathepsin D [CatD] genes) were immunoprecipitated with either an mSin3A antibody or an anti-*O*-GlcNAc antibody (RL-2). Since the catalytic activity of OGT is required for repression, the RL-2 antibody allowed us to precipitate the product of this catalysis, *O*-GlcNAcylated proteins bound to these promoters. Our result showed that, when the genes were silenced upon estrogen depletion, the *O*-GlcNAc level of the proteins on these promoters was increased, coincident with the elevated occupancy of mSin3A on the promoters (Figure 6). As a control, we observed no changes either in the protein *O*-GlcNAc level nor in the mSin3A level on the p21 promoter, a gene that is refractory to estrogen (Figure 6). This result suggests that mSin3A can recruit OGT to silenced genes to catalyze *O*-GlcNAcylation of promoter bound proteins under natural circumstances.

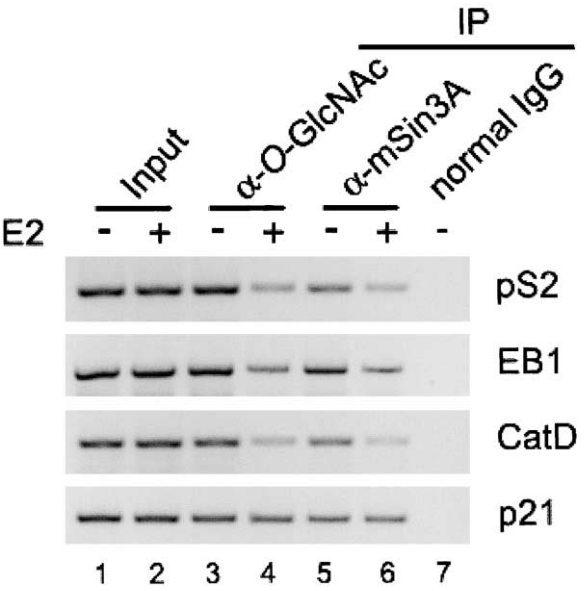

Figure 6. ChIP Analysis of Protein *O*-GlcNAcylation In Response to Gene Silencing

Soluble chromatin was prepared from estrogen-depleted MCF-7 cells with or without E2 treatment, followed by immunoprecipitation (IP) with anti-*O*-GlcNAc IgG (RL-2), anti-mSin3A IgG, or normal IgG. The DNA extracted from the respective immunoprecipitates was amplified using primers that cover the promoter regions of pS2, EB1, cathepsin D (CatD), and p21 genes. Input represents each PCR product from 2% of the preimmunoprecipitated DNA.

Discussion

Since Jackson and Tjian discovered the posttranslational modification of transcription factors by *O*-GlcNAc, the repertoire of transcription factors harboring this modification has been expanding rapidly (Hart, 1997; Jackson and Tjian, 1988). Nevertheless, it has been difficult to elucidate sole effects of this modification on transcriptional regulation because it often occurs reciprocally with phosphorylation (Wells et al., 2001). By focusing on a region in Sp1 activation domain that is subjected exclusively to *O*-GlcNAcylation, we have presented direct evidence that this modification per se can negatively regulate transcription factor activity (Yang et al., 2001). On the basis of this concept, we propose that OGT, the enzyme that catalyzes this modification, is involved in transcriptional repression. In this report, we show that OGT can inhibit both basal and Sp1-driven transcription. Remarkably, OGT physically associates with a corepressor complex involving mSin3A and HDAC1 and, through its TPR domain, directly contacts the mSin3A PAH4 domain. Coexpression of OGT and mSin3A in cells synergistically represses both basal and Sp1-activated transcription, indicating a functional interaction between OGT and the corepressor complex. Further analysis indicates that mSin3A and OGT might act via a HDAC-independent mechanism to repress transcription.

OGT May Be a Ubiquitous Regulator of Transcription

While there may be more than 2000 protein kinases encoded in the mammalian genome, each with its own subset of substrates, the OGT gene appears to be a single copy gene in metazoan genomes (Shafi et al., 2000). Yet, OGT protein catalyzes *O*-GlcNAcylation of numerous transcription factors and other intracellular proteins, implying that this enzyme displays much greater flexibility in recognizing its many substrates (Wells et al., 2001) than do the protein kinases. One hallmark of the OGT molecule is its tandem TPR motifs, which are very conserved throughout evolution and exist in a wide range of proteins (Lamb et al., 1995; Roos and Hanover, 2000). Distinct regions in the OGT TPR array might selectively contact a diverse set of transcription factors and modulate their glycosylation states after be-

ing attracted to promoters by a corepressor like mSin3A. This substrate flexibility, coupled with our observation that OGT is equally efficient in transcriptional repression when tethered to different points along the DNA upstream of a reporter gene, suggests that OGT is also flexible with regard to positioning on promoters. We believe that these flexible properties would strengthen the performance of OGT as a ubiquitous repressor of transcription. Since Sin3-HDAC corepressor complexes are involved in silencing of a large assortment of genes, the association between Sin3 and OGT could support a ubiquitous role in this process as well.

In addition to OGT, other corepressors have been found to contain clustered TPR motifs (Lamb et al., 1995). The Ssn6-Tup1 corepressor complex in yeast is a global transcriptional repressor that seems analogous to the OGT-mSin3A complex in many aspects. Ssn6 comprises 10 tandem TPR motifs, of which distinct combinations are responsible for interactions with its partner Tup1 and different DNA binding repressors (Tzamarias and Struhl, 1995). Ssn6-Tup1 can also span various distances along promoters for repression (Smith and Johnson, 2000). More importantly, it appears that Ssn6-Tup1 represses gene activity through two independent pathways: one involves targeting HDACs for localized histone deacetylation (Watson et al., 2000; Wu et al., 2001); a second involves interactions with components of the basal transcription machinery (Herschbach et al., 1994; Kuchin and Carlson, 1998). Similarly, our results indicate that OGT-mSin3A is likely to enlist HDACs as well as modulate the basal transcription machinery for gene silencing (Figure 7).

The chief difference between OGT-mSin3A and Ssn6-Tup1 may be that the OGT TPR domain is associated with an enzymatic activity while the Ssn6 is not. It is believed that Ssn6-Tup1 directly interferes with DNA-bound activators to block transactivation (Redd et al., 1996). If so, this requirement for direct and stoichiometric protein-protein interaction for gene silencing in yeast may be far less efficient and versatile than the metozoan mechanism utilizing a catalytically driven protein modification such as *O*-GlcNAcylation. Hence, we propose the model that OGT is recruited to the repressed gene to mask transcription factors by *O*-GlcNAc primarily at the transactivation domains and block their interactions with general transcription factors or coactivators (Yang et al., 2001). This model implies that OGT-Sin3 can act to repress gene transcription at substoichiometric levels. Indeed, our data indicate that the complex composed of OGT, mSin3A, and HDAC1 is substoichiometric. Furthermore, we did not detect Sp1 in this complex, which supports the notion that one OGT molecule could transiently bind to and inactivate multiple Sp1 molecules at promoters by the enzymatic mechanism. Finally, the activity of OGT in the repressor complex might be modulated by a variety of signals. OGT activity is regulated by the availability of its substrate, UDP-GlcNAc that is derived from glucose metabolism and there is evidence that the enzyme is phosphorylated (Kreppel et al., 1997). Hence, OGT-mediated repression may be subject to nutritional and signal-dependent regulation and in collaboration with histone deacetylation would ensure gene silencing in an efficient, specific, and regulated manner (Figure 7).

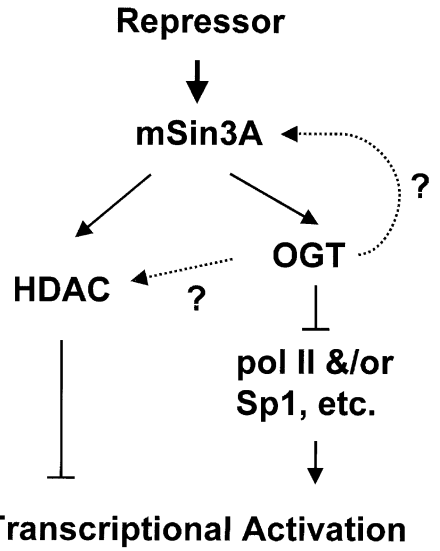

Figure 7. Model for Collaboration Between OGT and the HDAC Corepressor Complex for Gene Silencing

Sequence-specific repressors bind to the promoter of a gene and recruit the mSin3A-HDAC complex. mSin3A also recruits OGT to the promoter. OGT might inactivate activators and RNAPII that reside on the promoter by modifying these proteins with *O*-GlcNAc. For activators, *O*-GlcNAcylation might block their interactions with coactivators or general transcription factors. For RNAPII, sustained *O*-GlcNAcylation on its C-terminal tail might arrest transcriptional elongation and halt RNAPII recycling. In addition, OGT might regulate mSin3A and HDAC activities by *O*-GlcNAcylation, as indicated by the dotted lines with the question marks. These actions mediated by OGT are in collaboration with histone deacetylation by HDACs to ensure gene silencing in an efficient and specific manner.

OGT May Repress Transcription in a Gene-Specific Manner

The interplay between activation and repression allows genes to be expressed in elaborate spatial and temporal patterns. Activation is accomplished by ordered actions involving activators, chromatin remodeling factors, histone acetyltransferases, and the RNAPII holoenzyme (Agalioti et al., 2000; Cosma et al., 1999; Shang et al., 2000). Repression may involve the converse including repressor binding, chromatin remodeling, and histone deacetylation, although direct evidence has yet to emerge. A pertinent question is how activators and repressors that coexist on the promoter counteract each other when transitioning from activation to repression. Based on our studies of Sp1 *O*-GlcNAcylation (Roos et al., 1997; Yang et al., 2001), it is our hypothesis that OGT activity recruited by repressors via mSin3A might trigger the disassembly of the activation complex. The interruption of the hydrophobic interactions between these proteins by *O*-GlcNAc might shift the equilibrium of the gene expression from an active to a repressive state (Figure 7). While it has been generally believed repressed genes are inaccessible to activators and the basal transcription machinery, this view has been challenged by the recent finding that activators, TATA binding protein (TBP), RNAPII, and even Sp1 constitutively occupy genes in silenced chromatin (Sekinger and Gross, 2001). Moreover, in concert with our observation that proteins bound to the promoters of silenced estro-

gen-responsive genes are hyperglycosylated, it has been reported that the density of *O*-GlcNAcylated proteins is elevated in transcriptionally inactive regions along *Drosophila* polytene chromosomes (Kelly and Hart, 1989), implying an inverse relationship between transcription and *O*-GlcNAcylation. These findings are compatible with the idea that the *O*-GlcNAc modifications might render activators and RNAPII inactive, in spite of their occupancy of the promoter.

OGT probably has functions in addition to gene silencing. There is some evidence that OGT could be essential for recycling RNAPII during transcription. The CTD of RNAPII is *O*-GlcNAcylated and dephosphorylated during transcriptional initiation, whereas it is deglycosylated and phosphorylated during elongation (Dahmus, 1996; Kelly et al., 1993). In an actively transcribed gene, this cycle is intact such that OGT and CTD phosphatase may act cooperatively to recycle RNAPII into the preinitiation complex after one round of transcription. mSin3A may enable OGT to lock RNAPII on one side of this recycling process, contributing to gene silencing. This hypothesis may explain our observation that OGT and mSin3A cooperatively inhibit basal transcription (Figure 7).

The theme is emerging that phosphorylation, acetylation, methylation, ubiquitination, and ADP-ribosylation of histones and transcription factors coordinate transcription of genes (Hunter and Karin, 1992; Karin and Ben-Neriah, 2000; Pham and Sauer, 2000; Robzyk et al., 2000; Strahl and Allis, 2000; Berger, 2001; Chen et al., 2001; Mowen et al., 2001). Our studies suggest that *O*-GlcNAcylation plays a vital role in this theme as well.

Experimental Procedures

Plasmids

Mammalian expression plasmids pCMX-mSin3A and pCMX-Gal4-mSin3A were generously provided by R. Evans. pcDNA3-FLAG-HDAC1 was kindly provided by E. Seto. pcDNA3-OGT and G_5-Luc reporter construct were described previously (Yang et al., 2001). For protein expression in mammalian cells, the indicated mutants were subcloned into pcDNA3.1 (Invitrogen) while pGEX-2T (Amersham Pharmacia) was used for GST fusion protein expression in *E. coli*. The oligonucleotide for the triple HA epitope tag was synthesized and inserted into N terminus of OGT sequence in the pcDNA3.1 vector for expression of HA-OGT proteins. The sequence encoding the PAH4 region (amino acids 901–955) was removed from pCMX-Gal4-mSin3A using PCR to obtain the Gal4-mSin3A (ΔPAH4) construct. Detailed information regarding each construct is available upon request.

Cell Culture and Transient Transfection

Transient transfection of $HepG_2$ cells was performed by electroporation (Yang et al., 2001). Luciferase activities were assayed 48 hr later. Transfection efficiencies were normalized using a cotransfected β-galactosidase plasmid. Each transfection was done in triplicate and repeated 2–5 times.

GST Pull-Down Assays

All GST fusion proteins were expressed in *E. coli* and were purified as described previously (Su et al., 2000). [^{35}S]-labeled OGT, mSin3A, or AD-mSin3A deletion mutants were synthesized in vitro using the appropriate T7-based plasmid in a coupled transcription-translation system, TNT (Promega). [^{35}S]-labeled proteins were incubated with equal amounts of either immobilized GST or GST fusion proteins in binding buffer (50 mM Tris [pH 7.5], 10% glycerol, 100 mM NaCl, 0.1% NP-40, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and protease inhibitors) for 2 hr at 4°C. Beads were washed five times with the binding buffer. Bound proteins were eluted with 1 × SDS-PAGE buffer, separated by SDS-PAGE, and visualized by fluorography.

Coimmunoprecipitation Assay

Cos-7 cells were transfected by Targefect F-2 (Targeting Systems, San Diego, CA) with 16 μg of expression vectors (8 μg each) encoding the indicated HA epitope-tagged OGT and mSin3A proteins. 48 hr later, cells were harvested in lysis buffer (50 mM Tris [pH 8.0], 20% glycerol, 500 mM NaCl, 0.5% NP-40, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, and protease inhibitors). Cellular debris was removed by centrifugation. Supernatants were diluted with 4 × volumes of dilution buffer (50 mM Tris [pH 7.5], 10% glycerol, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, and protease inhibitors), followed by immunoprecipitation at 4°C with α-HA antibody (12CA5, Roche) and protein G-Sepharose 4 Fast Flow (Amersham Pharmacia). Precipitates were washed five times with LS buffer (50 mM Tris [pH 7.5], 10% glycerol, 100 mM NaCl, 0.1% NP-40, 1 mM EDTA), resolved on SDS-PAGE, and Western blotted using an α-HA antibody (12CA5), α-mSin3A antibody (K-20, Santa Cruz), α-HDAC1 antibody (H-11, Santa Cruz), α-HDAC4 antibody (Upstate), and α-Sp1 antiserum (Su et al., 1999), respectively.

Analysis of Protein *O*-GlcNAcylation

Cos-7 cells were transiently transfected with pCMX-mSin3A or pcDNA3-FLAG-HDAC1, respectively. After 18 hr, cells were infected with recombinant vaccinia virus VTF7-3 encoding T7 RNA polymerase. 24 hr after infection, cells were lysed then immunoprecipitated at 4°C with α-mSin3A or α-FLAG M2 antibody (Sigma). Precipitates were washed five times, resolved on SDS-PAGE, and Western blotted. The membranes were probed with α-*O*-GlcNAc antibody (Affinity Bioreagents) and then reprobed with α-mSin3A or α-FLAG M2 antibodies.

Chromatin Immunoprecipitation (ChIP)

MCF-7 cells were grown to 95% confluence in DMEM supplemented with 10% charcoal-dextran stripped FBS for at least 3 days before treatment with 100 nM 17β-estrodiol (E2, Sigma) for 2 hr. Cells were crosslinked with 1% formaldehyde at room temperature for 10 min. Cell lysates were prepared as described previously (Shang et al., 2000) and then were sonicated. Cell debris was removed by centrifugation. Supernatants were precleared with 20 μg sheared salmon sperm DNA, 5 μg normal IgG, and 50 μl protein G-sepharose for 2 hr at 4°C. Immunoprecipitations were performed overnight at 4°C with RL-2 or α-mSin3A antibodies. Immunoprecipitates were washed and eluted as described (Shang et al., 2000), then heated at 65°C for 6 hr to reverse the formaldehyde crosslinking. DNA fragments were purified with DNA Clean & Concentrator-5 (Zymo Research). Quantitative PCR was performed with 1 μl from a 50 μl DNA extraction for 30–32 cycles. Linearity of PCR amplification for the indicated genes was demonstrated by serial 3-fold dilutions of the input DNA.

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