Drosophila O-GlcNAc transferase (OGT) is encoded by the *Polycomb* group (PcG) gene, *super sex combs* (*sxc*)

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***O*-linked *N*-acetylglucosamine transferase (OGT) reversibly modifies serine and threonine residues of many intracellular proteins with a single *β*-*O*-linked *N*-acetylglucosamine residue (*O*-GlcNAc), and has been implicated in insulin signaling, neurodegenerative disease, cellular stress response, and other important processes in mammals. OGT also glycosylates RNA polymerase II and various transcription factors, which suggests that it might be directly involved in transcriptional regulation. We report here that the *Drosophila* OGT is encoded by the Polycomb group (PcG) gene, *super sex combs* (*sxc*). Furthermore, major sites of *O*-GlcNAc modification on polytene chromosomes correspond to PcG protein binding sites. Our results thus suggest a direct role for *O*-linked glycosylation by OGT in PcG-mediated epigenetic gene silencing, which is important in developmental regulation, stem cell maintenance, genomic imprinting, and cancer. In addition, we observe rescue of *sxc* lethality by a human *Ogt* cDNA transgene; thus *Drosophila* may provide an ideal model to study important functional roles of OGT in mammals.**

epigenetic | gene silencing | *O*-glycosylation | glycosyl transferase

The enzyme OGT catalyzes the addition of a single *O*-GlcNAc residue to serine and threonine residues of a large number of proteins with diverse cellular functions (1). *O*-GlcNAc is a reversible posttranslational modification; another enzyme, *O*-GlcNAcase (OGA), can remove the sugar. In mice, the single *Ogt* gene is required for viability (2). Overexpression or knockdown of *Ogt* has implicated it and/or the *O*-GlcNAc posttranslational modification in a wide range of key signaling pathways and cellular processes, including roles in insulin signaling/diabetes (3), cell−cycle regulation (4), stress response (5), and immune cell regulation (6). Intriguingly, OGT also modifies unphosphorylated Pol II (Pol IIA) and a number of transcription factors (7); however, demonstrating a clear functional role for *O*-linked glycosylation by OGT in transcriptional regulation has proven elusive (1).

While working on a functional annotation of centric heterochromatin of *Drosophila melanogaster*, we noted that the *Ogt* gene was in a region of chromosome 2R that includes the Polycomb group (PcG) gene *super sex combs* (*sxc*) (8). Initially characterized in *Drosophila* as regulators of Hox gene expression along the anteroposterior axis in early development, PcG proteins form multiprotein complexes that epigenetically regulate an extensive list of target genes in animals and plants. PcG proteins play key roles in developmental regulation, stem cell maintenance, genomic imprinting, and cancer, although the mechanism of this epigenetic regulation is not yet completely clear (reviewed in refs. 9–12).

The *sxc* gene has not been well characterized, in large part because it is localized within centric heterochromatin, which has proven more difficult to map and sequence than euchromatin (13). As observed for mutations in other *PcG* genes, loss of *sxc* function results in derepression of *Hox* genes (8, 10), with resulting homeotic transformations, including the appearance of ectopic sex combs on mesothoracic (T2) and/or metathoracic (T3) legs. Unlike most other *PcG* genes however, *sxc* alleles are recessive and exhibit a late lethal phase i.e., *sxc*-null flies die as pharate adults, although early derepression of *Hox* genes is observed in embryos (8). Data from pole-cell transplantation experiments have confirmed that *sxc* is essential during embryogenesis and that this requirement is fulfilled by maternally derived sxc^{+} product (8). Finally, *sxc* mutations enhance the phenotypes of mutations in other *PcG* genes (14).

Here we report that the *sxc* gene encodes the *Drosophila* OGT protein: sxc mutant DNA shows corresponding lesions in the *OGT* gene, and expression of a *Drosophila* or even human ogt transgene rescues sxc mutant flies. We also observe extensive overlap between sites of *O*-GlcNAc protein modification and sites of PcG protein binding on polytene chromosomes and in chromatin immunoprecipitation (ChIP) experiments, further suggesting that OGT activity has an important role in PcG gene silencing. Our results thus point to an intriguing link between OGT activity and epigenetic gene regulation by PcG proteins, and suggest that *Drosophila* may provide a powerful model system to further study the roles of OGT in important cellular processes.

Results

Mapping of the *sxc* Locus to the Region Containing *Ogt*. The *sxc* gene was originally mapped to a proximal segment of chromosome 2R heterochromatin; further genetic analysis (see supporting information (*SI*) Fig. S1, *SI Text*) suggested that *sxc* corresponded to 1 of the 7 genes located between *Nipped-A* and CG42345 (overlapping the dotted line in Fig. S1). Because *sxc* is involved in gene repression (8), we felt that the best candidates were CG2682 (*d4*), CG10392 (*Ogt*), and CG10417 (which encodes a protein phosphatase). We also favoured the idea that *sxc* might encode an enzyme, because this would be more consistent with the observation that *sxc* mutations act in a recessive manner: both gene copies must be inactivated to show a phenotype, whereas most *PcG* genes are dominant modifiers. Additionally, attempts to rescue *sxc* lethality using a UAS-cDNA transgene for *d4* were unsuccessful, suggesting that *sxc* and *d4* are not equivalent (D. Sinclair, unpublished results). Because *sxc* is fairly mutable (8), and because the *Ogt* gene product is larger than that of CG10417 (http://flybase.org/), and also given the potential

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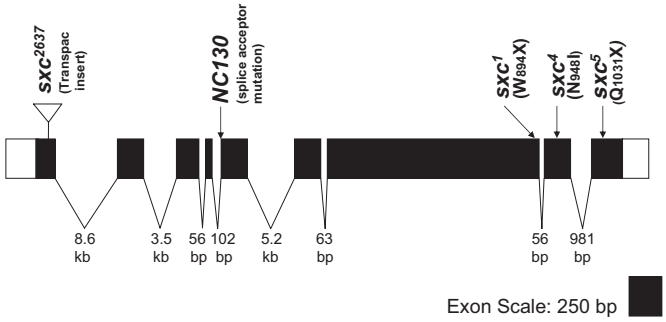

Fig. 1. Schematic map of the *Drosophila Ogt* gene and *sxc* mutations. The solid boxes represent exons with black (protein coding) and white (5′ and 3′ noncoding) regions. The angled lines below indicate the introns and their sizes. The nature and position of *sxc* mutations is indicated above the lines; X indicates a stop codon.

link between OGT signaling and *PcG* function, we decided to focus first on the possibility that *sxc* corresponded to *Ogt*.

Mutations in the *PcG* Gene *sxc* Show Corresponding Lesions in the *ogt* Gene and OGT Protein. In an attempt to link the *Drosophila Ogt* gene to *sxc*, we isolated genomic DNA from mutant *sxc* embryos, and used gene-specific primers to amplify and sequence across portions of the *Ogt* genomic locus (15). The results, summarized in Fig. 1, strongly suggest that *sxc* encodes the OGT protein. In sxc^{1}/sxc^{1} and sxc^{5}/sxc^{5} embryos, we identified nonsense mutations in *Ogt* that would result in expression of truncated enzyme with a resulting loss of function, while sxc^{4}/sxc^{4} embryos showed a missense mutation of a highly conserved N residue to I, which is located close to the active site (16). In sxc^{NC130}/sxc^{NC130} embryos we found a splice acceptor mutation (AG to AA) that should result in an aberrant transcript and production of truncated enzyme. sxc^{2637}/sxc^{2637} embryos have a large transpac transposable element insertion within the first exon of *Ogt*, which would disrupt gene function (see *SI*). Another mutation, *Df(2R)NC31*, is a chromosomal deficiency (Df) that results in deletion of the entire *Ogt* coding region (data not shown).

To demonstrate that the *sxc* mutations affected OGT protein, we examined third instar mutant larvae, in which maternally contributed protein or mRNA have dropped to low levels, to evaluate changes in OGT protein levels and enzyme activity. Western blot analysis shows a complete loss of OGT protein and OGT activity in sxc^{2637}/sxc^{2637} mutant larvae (Fig. 2*A* and *B*) and in other mutant combinations predicted to express no protein (Figs. S2 and S3). The missense allele sxc^{4} does show an immunoreactive protein of the expected size for OGT, yet enzyme activity is nearly completely absent (Figs. S2 and S3). While we were unable to find a DNA lesion in sxc^{3} mutants, we confirmed that it is an OGT null by Western blot and enzyme activity assays (Fig. S2 and S3). Because *sxc* mutations affect expression and/or activity of OGT protein in vivo, these data further suggest that *sxc* encodes OGT.

***sxc* Mutations Are Rescued by *ogt* Transgenes.** If the *sxc* gene encodes OGT, then a *Drosophila Ogt* transgene should rescue flies carrying *sxc* mutations. Consistent with this view, a pUAST-*Ogt* transgene that is ubiquitously expressed under the regulation of a tubulin-GAL4 driver rescues *sxc* lethality (Table 1). We recovered 64 surviving sxc^{3}/sxc^{2637} transheterozygotes carrying the *Drosophila Ogt* transgene, compared to zero expected and observed in the absence of the transgene (no rescue, Table 1). The rescue of *sxc* lethality by the *Ogt* transgene establishes that *sxc* and *Ogt* are the same locus.

Parallel experiments show that knockdown of *Ogt* gene expression using RNAi constructs reduces OGT protein expression significantly (Fig. 2*C*). As expected, many of these *Ogt* RNAi lines enhance lethality in *sxc* heterozygotes (Fig. S4) and in some cases, dead male pharate adults, dissected from pupal cases, exhibited ectopic sex combs, a phenotype indistinguishable from that observed in *sxc/sxc* mutant flies (data not shown).

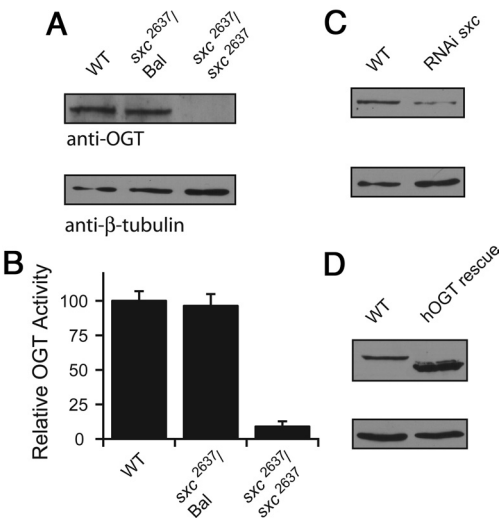

Fig. 2. Measurements of OGT protein levels and enzyme activity. (*A*) Western blot analysis of homogenates of larvae containing 2 functional copies of the *sxc* gene (wild type, lane1), 1 copy (sxc^{2637}/Balancer (Bal), lane 2), or no copies (sxc^{2637}/sxc^{2637} homozygous, lane 3). (*B*) OGT enzymatic activity in extracts from wild-type, sxc^{2637}/Bal, and sxc^{2637}/sxc^{2637} larvae were assayed using recombinant p62 as the acceptor substrate. Activity is reported as a percentage of activity relative to that found for wild-type fly larvae extracts. (*C*) Western blot analysis of homogenates of wild-type flies (lane 1) versus those carrying a tubulin-GAL4 driven *Ogt* RNAi transgene (lane 2). (*D*) Western blot analysis of adult wild-type (lane 1) and sxc^{3}/sxc^{2637} mutant flies rescued by a tubulin-GAL4 driven human *Ogt* transgene (lane 2). For Western blots, all extracts were probed for OGT (*Upper*) using rabbit anti-OGT Ab (H-300) and also for β-tubulin using a mouse anti-β-tublin mAb (E7) to ensure similar loading of samples (*Lower*).

Human and fly OGT proteins show 77% identity and 86% similarity, based upon protein sequence alignment (data not shown). We wanted to determine whether this functional conservation was sufficient for a human *Ogt* cDNA transgene to rescue *sxc* lethality. The human *Ogt* transgene also rescues *sxc* mutants, with 82 rescued progeny compared to zero expected in the absence of the transgene (Table 2). The large number (82 versus zero) and similar proportion (roughly 20%) of rescued relative to total progeny, suggest that the human gene rescues *sxc* lethality as efficiently as its fly counterpart, and likely functions similarly in flies and humans. For both the *Drosophila* and human transgenes, rescued males and females were fertile and appeared normal, except that the majority had small amounts of ectopic wing vein material radiating away from the posterior cross vein (between L4 and L5). In Fig. 2*D*, Western blot analysis confirms corresponding expression of the slightly smaller human OGT protein in these transgenic flies. These data indicate that *Ogt* likely has analogous functional and regulatory roles in development in *Drosophila* and mammals, making *Drosophila* an attractive model for studying OGT function.

Colocalization of *O*-GlcNAc Protein Modification and PcG Binding on Polytene Chromosomes. To determine whether the addition of *O*-GlcNAc residues by OGT might act in concert with PcG protein binding to downstream target genes, we used an anti-*O*-GlcNAc antibody for in situ analysis of polytene chromosomes. This antibody was chosen because the available anti-OGT

Table 1. Rescue with *Drosophila Ogt* cDNA transgene

Parental genotype	F1 genotype			Total
	CyRoi	*CyRoi TM3*	sxc^{3}/sxc^{2637}	
sxc^{3}/*CyRoi; tub-GAL4/TM3 Ser* X sxc^{2637}/*CyO* (mutant progeny lack transgene)	85	41	0 (no rescue)	126
sxc^{3}/*CyRoi; tub-GAL4/TM3 Ser* X sxc^{2637}/*CyRoi; UAS-D. melanogaster Ogt cDNA/TM3 Sb* (mutant progeny have transgene)	112	129	64 (rescue)	305

Ubiquitous expression of *D. melanogaster Ogt* cDNA rescues *D. melanogaster* sxc^{3}/sxc^{2637} mutants. The surviving sxc^{3}/sxc^{2637} transheterozygotes (64) are fertile and have ectopic wing vein material.

antibody used for Western blot analysis was not suitable for immunohistochemistry or chromatin immunoprecipitation (ChIP) analysis (data not shown). This anti-*O*-GlcNAc antibody binds to a limited number of sites on polytene chromosomes, and these sites show extensive overlap with major binding sites of the PcG protein Polyhomeotic (Ph). Fig. 3 shows *O*-GlcNAc binding sites on chromosome 3R at the bithorax (BX-C) and Antennapedia complexes, well-known *PcG* targets (9–12), and at other discrete sites. We obtained similar results for Polycomb (Pc) protein (data not shown). This result is consistent with recent genomewide analyses showing that PcG proteins, including Pc and Ph, bind at the same sites (17, 18), and suggests that 1 or more PcG proteins could be candidate substrates.

The resolution of in situ hybridization is not sufficient to show that OGT acts at the known sites of PcG protein binding, the PcG response elements (PREs). To show this more definitively, we used chromatin immunoprecipitation (ChIP) to assay the presence of *O*-GlcNAc at the bithoraxoid PRE of *Ultrabithorax* (*Ubx*), a well-defined component of BX-C which has been extensively characterized (19, 20). The *O*-GlcNAc binding pattern is strongest in the middle of the PRE, consistent with the previously reported positioning of PcG protein binding (19, 20) and with our own data for binding of Polyhomeotic (Fig. 4*B*). Moreover, binding of this anti-*O*-GlcNAc antibody was abolished in $sxc^{3}/Df(2R)NC31$ mutants (Fig. 4*C*), which demonstrates that the specificity of the antibody is limited to *O*-GlcNAc-containing epitopes, indicative of OGT activity. We also observed that, while *O*-GlcNAc signal is absent, binding of Pc and Ph is unaffected in *sxc*/*sxc* mutants (Fig. S5), suggesting that recruitment of PcG complexes to polytene chromosomes does not depend significantly upon OGT activity. This result again indicates that the detected signal is OGT-dependent and specific to *O*-GlcNAc residues.

Discussion

Our results clearly demonstrate that the PcG gene *sxc* encodes the *Drosophila* OGT enzyme. Mutations in *sxc* cause homeotic phenotypes (9) because of derepression of homeotic loci (21, 22) and enhance the phenotypes of some PcG mutations (14). Together with the existing genetic and molecular data, our polytene and ChIP binding data show that OGT acts directly at PcG targets in chromatin but does not have a direct role in recruiting PcG protein to chromatin: *O*-GlcNAc modification, but not Pc/Ph binding, is abolished at PcG target sites in *sxc*/*sxc* mutants. We therefore hypothesize that *O*-glycosylation by OGT has a primary role in gene silencing by PcG complexes.

The experiments with polytene chromosomes required very high titers of antibody, leading us to believe that this antibody recognizes predominantly major sites with relatively high levels of *O*-GlcNAc modification; again, the absence of signal in *sxc*/*sxc* mutants (Fig. S5 and S4*C*) confirms that this modification is OGT dependent. We also observe staining representing lower background levels of *O*-GlcNAc uniformly across the chromosomes (Fig. 3). The latter result is consistent with a previous report showing *O*-GlcNAc-modified chromatin proteins ubiquitously localized on polytene chromosomes (23).

PcG proteins regulate many targets, including genes encoding proteins that function throughout development (17, 18). Some of the pleiotropic phenotypes of *Ogt* mutants may thus be a consequence of improper silencing of PcG targets, although our data do not rule out other roles for OGT and *O*-GlcNAc modification in the many important cellular processes linked to OGT function. Homozygous *sxc*/*sxc* mutants survive over 5 days until late larval or pharate adult (but die in pupal cases) stages, presumably because maternally contributed *sxc* mRNA or protein, deposited in the fertilized egg, compensates for the loss of zygotic *sxc* function. This suggests that most cellular processes are relatively insensitive to gradual loss of OGT in development.

Table 2. Rescue with human *Ogt* cDNA transgene

Parental genotype	F1 genotype			Total
	CyRoi	*CyRoi TM3*	sxc^{3}/sxc^{2637}	
sxc^{3}/*CyRoi; tub-GAL4/TM3 Ser* X sxc^{2637}/*CyO* (mutant progeny lack transgene)	85	41	0 (no rescue)	126
sxc^{3}/*CyRoi; tub-GAL4/TM3 Ser* X sxc^{2637}/*CyRoi; UAS-Hs Ogt cDNA/UAS-Hs Ogt cDNA* (mutant progeny have transgene)	191	128	82 (rescue)	401

Ubiquitous expression of human *Ogt* cDNA rescues *D. melanogaster* sxc^{3}/sxc^{2637} mutants. The surviving sxc^{3}/sxc^{2637} transheterozygotes (82) are fertile and have ectopic wing vein material.

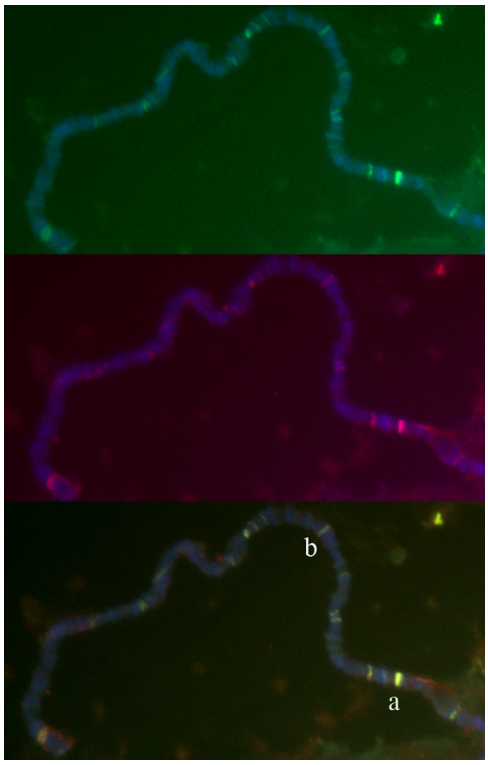

Fig. 3. Overlapping binding sites for anti-*O*-GlcNAc and anti-Ph antibodies on chromosome 3R. Polytene chromosomes were reacted with antibodies to *O*-GlcNAc (*Top*) and Ph (*Middle*). These images were superimposed on chromosomes stained with DAPI, and on each other (*Bottom*). The locations of the Antennapedia complex (''a'') and bithorax (BX-C) complex (''b''), well-characterized PcG targets, are indicated in *Bottom*.

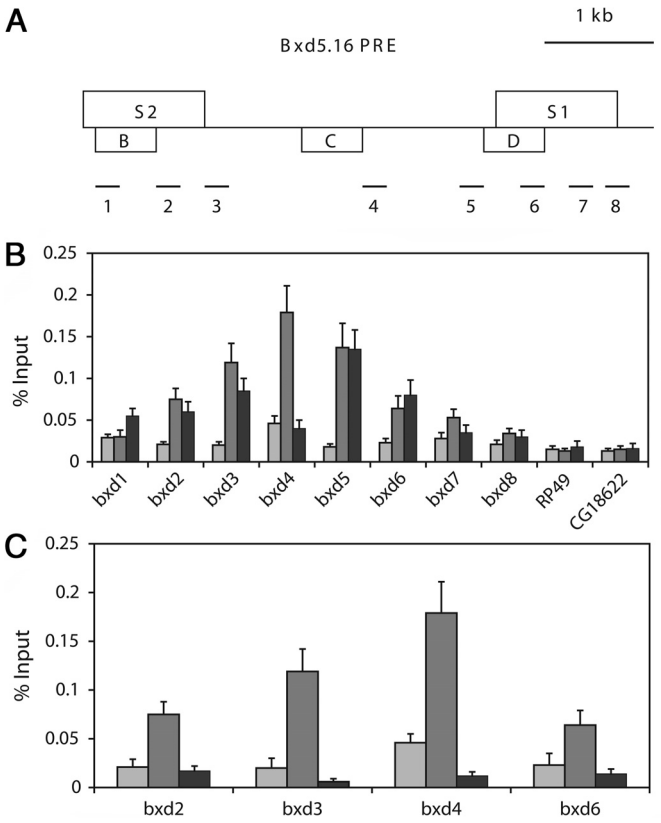

Fig. 4. ChIP analysis of *O*-GlcNAc modifications at the BX-C locus. (*A*) A map of the bxd PRE and location of 8 primer pairs used to quantitate binding is shown. (*B*) Percentage of input binding for each primer pair in embryos is shown. Light gray, IgG control; dark gray, GlcNAc; black, Ph. Negative controls, *rp49* (*ribosomal protein 49*), CG18622 (a gene just outside AbdB of the BX-C that doesn't bind PcG proteins). (*C*) *O*-GlcNAc binding is absent in polytene chromosomes of sxc^{-}/sxc^{-} mutants. Light gray, IgG; dark gray, GlcNAc WT; black, GlcNAc $sxc^{-/-}$ mutant.

Interestingly however, inappropriate derepression of *Hox* genes is detectable by 4.5 h postfertilization in *sxc/sxc* embryos, similar to that observed for other PcG mutations (22). Furthermore, we have observed almost complete embryonic lethality when *ogt* RNAi transgene expression is controlled by a Gal4 driver (69B), which drives expression in the embryonic epidermis (results not shown). Thus silencing of *Hox* genes is very sensitive to *sxc* mutations and to RNAi-induced reductions in ogt gene expression; these results suggest an early and essential role for *O*-GlcNAc modification of proteins in PcG silencing.

To convincingly address how OGT mediates PcG silencing will require extensive further study. The first challenge will be to obtain a complete inventory of chromatin-associated proteins modified with *O*-GlcNAc at PcG target sites, because these candidate substrates could provide the functional link between OGT activity and PcG silencing. One plausible candidate target is the PcG protein, Pleiohomeotic (Pho), a DNA-binding protein believed to play a key role in recruiting PcG complexes to specific PcG binding sites (reviewed in ref. 24). *O*-GlcNAc modification of YY1, the mammalian orthologue of *Drosophila* Pho, does not affect its affinity for DNA but does alter its interaction with protein partners (25). Pho shares almost complete amino acid sequence identity with its mammalian orthologue YY1, in key functional regions including the Zn finger and spacer domains (26). A number of transcription factors, and the C-terminal domain (CTD) region of the large subunit RNA polymerase II itself, are also modified by *O*-GlcNAc (1, 7), as is a protein associated with E(Pc), another PcG protein (27). Other potential substrates may yet be identified; for example, OGT associates with a number of other cellular proteins, including the mSin3A chromatin-modifying complex (28) and the cell proliferation factor HCF-1 (29).

Interestingly, it has recently been reported that OGT interacts with MLL-5 and subsequent *O*-GlcNAc modification is required for H3K4 methylation and response to retinoic acid (30). Moreover, Chalkley et al. (31) have recently characterized a number of *O*-GlcNAc-modified members of gene repressor complexes, including a protein related to Polyhomeotic (mammalian polyhomeotic-like protein 3). These results provide corroborative evidence that *O*-GlcNAc modifications may have key roles in gene regulation and identify more putative OGT substrates. A second major challenge will be to establish a direct, mechanistic link between *O*-GlcNAc modification of target substrate(s) and subsequent PcG silencing.

Finally, both mice (2) and flies require *Ogt* function for viability; our demonstration of rescue of *sxc* lethality by a human *Ogt* cDNA transgene implies that key roles for OGT first demonstrated in humans—signaling, stress response, and others—may also be relevant in flies. *Drosophila* may therefore provide a powerful model for further work on elucidating the role of *Ogt* in critically important cellular processes.

Materials and Methods

***Drosophila* Stocks and Culture Conditions.** Flies were grown at 25 °C on cornmeal-molasses-yeast medium supplemented with the mold inhibitors tegosept and propionic acid. Six different *sxc* alleles were used in the current study. The origins of the EMS-induced alleles, sxc^{1}, sxc^{3}, sxc^{4}, and sxc^{5}, are described in Ingham (8); sxc^{1} was induced on a second chromosome bearing

the markers *cn*, *bw*, and *sp* and the other 3 alleles were induced on an isogenic second chromosome bearing the markers *b*, *pr*, *cn*, and *bw*. sxc^{NC130}, an EMS-induced allele obtained from the Bloomington Stock Center, was generated more recently using a second chromosome bearing the markers *cn* and *bw* (32); its status as an *sxc* allele was confirmed by complementation (D. Sinclair, unpublished results). sxc^{2637}, also obtained from the Bloomington Stock Center, was generated in an extensive *P*-element-based gene disruption study (33) and was originally designated *l (2)02637*. See *SI* for more on its genetic and molecular characterization. Finally, *Df(2R)NC31*, an EMS-induced mutation that was previously defined as a *Nipped-A* allele (32), was kindly provided by D. Dorsett. It was more recently determined to be a deletion for *Nipped-A*, *sxc*, and several other adjacent genes in proximal 2R (D. Sinclair, unpublished results).

Single Embryo PCR. *super sex combs* (*sxc*) mutant alleles were balanced over CyO *Kr-GAL4 UAS-GFP or CyO twi-GAL4 UAS-GFP* chromosomes. Genomic DNA was isolated from individual embryos of each strain and PCR was used (15) to identify samples originating from homozygous embryos containing *sxc* defects. Primers were designed to amplify the GFP transgene (330-bp fragment) and an X-linked gene used to evaluate DNA integrity, *Grp84* (215-bp fragment) as previously described (34). DNA specimens for which PCR amplification detected the presence of *Grp84* and the absence of the *GFP* transgene were used in subsequent analysis. Using DNA from homozygous embryos, PCR was performed using gene-specific primers designed to anneal to regions flanking the exons of the *ogt* gene.

Sequence Analysis of Mutant *O*-Glycosyltransferase (Ogt) Genes. DNA sequencing using *ogt* gene-specific primers (see Table S1 for details) was done by Macrogen Inc. Wild-type *ogt* sequence for exons 1 to 9, including all splice junctions, was downloaded from the FlyBase Web site (http://www.flybase.org). Sequences from *sxc* mutants were compared to wild-type sequence using the Basic Local Alignment and Search Tool (BLAST) algorithm for 2 sequences (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). Default BLAST settings were used, with the exception of leaving low-complexity sequences unmasked. ORFs of sequences with changes were translated using ApE Plasmid Editor (ApE version 1.11 by M. Wayne Davis), and the subsequent protein sequences were aligned with wild-type *D. melanogaster Ogt* sequence using BLASTP to identify any changes at the amino acid level. Multiple protein sequence alignments were generated with BioEdit (BioEdit version 7.0.5 by Tom Hall).

Assays of OGT Protein Levels and Enzyme Activity. Third instar (L3) larvae were prepared from transheterozygous lines, generated in genetic crosses that combine 2 different *sxc* alleles, or combining an *sxc* mutation over *Df(2R)NC31*, which also lacks *sxc* function. Generating *sxc* transheterozygotes was necessary because most *sxc* alleles (except sxc^{2637}) carry second-site mutations; when present in 2 copies, these other mutations cause death at earlier stages of development. Forty milligrams of L3 larvae (approximately 40 larvae) were homogenized in 1 mL of lysis buffer (50 mM NaH_2PO_4, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM EDTA, and 1 mM NButGT, pH 7.4) using a tissue homogenizer (T-18 Ultra-Turrax). Insoluble cell debris was removed by centrifugation at 17,900 $\times$ *g* for 20 min and the resulting supernatant was used for Western blots.

OGT protein levels were determined via Western blotting: membrane was probed with either anti-OGT polyclonal IgG (H-300, Santa Cruz Biotechnology) at a dilution of 1:2,500 or a control anti-β-tubulin mAb IgG (Developmental Studies Hybridoma Bank) at a dilution of 1:2,000, followed by incubation with the appropriate HRP conjugated secondary antibody (Santa Cruz Biotechnology) and development with SuperSignal West Pico Chemiluminescence substrate (Pierce).

For OGT activity assays, larvae were homogenized as above, except that detergents were left out of the lysis buffer. These extracts were incubated with protein A/G-agarose beads (40 μL Calbiochem) with prebound anti-OGT antibody. After incubation and washes, OGT protein bound to the beads was assayed by adding 50 μL of recombinant p62 (1 μM) in 50 mM Tris, 150 mM NaCl, 0.5% Nonidet P-40, 12.5 mM $MgCl_2$, 1 mM β-ME, and 700 nM [^{3}H]-UDP-GlcNAc (0.1 μCi/μL) (Perkin–Elmer). Following incubation and washing in PBS, 50 μL of this combined supernatant was spotted onto a small piece (1.5 $\times$ 2.5 cm) of nitrocellulose membrane (Bio-Rad) in duplicate and allowed to dry. The membranes were then washed extensively with 5 large volumes of PBS. Levels of incorporated [^{3}H]-UDP-GlcNAc were then determined by scintillation counting. All assays were done in triplicate and error bars represent the SD.

Transgene Constructs. The pP{UAST-*Dm Ogt*} cDNA construct used for phenotypic rescue was generated as follows: an *Ogt* cDNA was prepared from isolated mRNA by RT-PCR. BglII and XbaI restriction sites were introduced via the indicated reverse-transcription PCR primers at the 5′ (GCGCAGATCTAAATCGTATTAACAGCTGAGTGG) and 3′ (GCCGTCTAGATTCTTATTATATATCGTATAAC) ends of the *Ogt* cDNA, respectively, to directionally clone the cDNA insert into the pUAST polylinker.

The pP{UAST-Hs *Ogt*} cDNA construct was generated by subcloning the human *Ogt* cDNA (obtained from the ATCC; cDNA clone MGC: 39117 IMAGE: 5017795, accession no. BC038180) into the NotI and EcoRI sites of the *Drosophila* germline transformation vector pUAST.

Stable *Ogt* RNAi lines were generated by PCR amplification and cloning of the region of the *Drosophila Ogt* cDNA encoding the 420th to 640th amino acid residues into the pSympUAST vector (35). Primers used were OGTRNAiEcoR1For: GGAAGCTATTA*GAATTC*AACCCACATTTGC and OGTRNAiBglIIRev: GC*AGATCT*CGACTGATTTTGTGTCGAAATGTTG.

Trangenic *Drosophila* were generated in a w^{1118} background by microinjection into fly embryos (BestGene, Inc.) using standard methods (36).

Rescue Crosses. For testing phenotypic rescue with the *D. melanogaster UAS-Ogt* transgene, sxc^{2637}*/CyRoi; UAS Dmel Ogt* cDNA/*TM3 Sb* males and sxc^{3}*/CyRoi; tub-GAL4/TM3 Ser* females were generated using standard genetic methods. For testing rescue with the human *UAS-Ogt* cDNA transgene, sxc^{3}*/CyRoi; tub-GAL4/TM3 Ser* males and sxc^{2637}*/CyRoi; UAS-HsOgt* cDNA/*UAS-HsOgt* cDNA females were generated and crossed together. In both cases, progeny genotypes were identified via visible phenotypic markers and counted until the 18th day following cross set-up. There is some variability in proportions of genotypes recovered, which are likely attributable to effects of combining different balancer chromosomes with multiple rearrangements. The proportion of rescued to total progeny (64/305 versus 82/401) is similar for both constructs. All *UAS* and *GAL4* constructs are located on the third chromosomes.

RNAi Crosses and Determination of Relative Viability. sxc^{3}*/Cy*, *Roi*; *Tub GAL4/TM3, Ser* females were crossed separately to 10 different *Ogt* RNAi second or third chromosome transgenic lines designated 1m–10m. In some cases, the RNAi transgenes were heterozygous with either *CyO* or *TM3*, *Sb* (the inserts were recessive lethal); in other cases, the RNAi transgenes were homozygous. The F_1 offspring (n = 251–600) were examined for relative viability of the 2 diagnostic classes: (1) *Ogt* RNAi transgene; *Tub GAL4*; sxc^{3} (RNAi driven in an sxc^{3} mutant background) and (2) *Ogt* RNAi transgene; *Tub GAL4*; *Balancer* (RNAi driven in an sxc^{+} background). Relative viability for each cross was determined by the ratio: [Number of diagnostic class flies]/[Number of *Ogt* RNAi transgene; sxc^{3}; *Balancer* flies]. In addition, for each cross, male pharate adults were examined visually for ectopic sex combs, the most obvious diagnostic of *sxc* homeosis.

Immunohistochemistry of Polytene Chromosomes. A monoclonal antibody to *O*-GlcNAc (Abcam 2735) was used at a dilution of 1:10 to 1:50. Turner et al. (37) have shown that this antibody is specific for *O*-GlcNAc modification; similar experiments verify this specificity in the staining of polytene chromosomes (Fig. S6) and our results showing the absence of antibody binding in sxc/sxc mutants provide further corroboration. A rabbit antibody to the proximal isoform of Polyhomeotic described previously (38) was used at a dilution of 1:175, and an antibody to Pc (a generous gift of Renato Paro) was used at a dilution of 1:75. Polytene chromosomes were prepared and subjected to immunohistochemistry as previously described (39). DNA was stained with 4,6-diamidion-2-phenylindole (DAPI) at 0.01 mg/mL. Antibody binding was detected with goat anti-mouse labeled with Alexa Fluor 568 and goat anti-rabbit labeled with Alexa Fluor 488 (Molecular Probes) used at a dilution of 1:100. Black and white images were collected by a CCD camera mounted on a Zeiss Axiophot microscope. These images were pseudocolored and superimposed using Adobe Photoshop.

Chromatin Immunoprecipitation. Chromatin immunoprecipitation was carried out as described (40) using lysates from 10- to 16-h embryos or from salivary glands. For each immunoprecipitation we used lysate containing 25 ng of DNA. The primary antibody was incubated with the lysate overnight at 4 °C and collected with agarose beads bound to protein A/G. After extensive washing, DNA in 10 mM Tris-HCl pH8.0, 1 mM EDTA was eluted from the beads using Chelex 100 resin followed by 10 min of heating at 95 °C. The amount of immunoprecipitated DNA was determined using Sybr Green and quantitative PCR. Data with the antibody of interest were compared to data using mixed Ig (IgG) used as a control for nonspecific binding and are expressed as percentage of input DNA. For each antibody, 3 independent immunoprecipi-

tations were carried out, and for each immunoprecipitation we performed 2 technical replicates. See *SI* for more on primers used.

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