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PRIMER

The role of O-GlcNAcylation in development

Ignacy Czajewski[1] and Daan M. F. van Aalten[1,2,3,*]

ABSTRACT

O-GlcNAcylation is a dynamic post-translational modification performed by two opposing enzymes: O-GlcNAc transferase and O-GlcNAcase. O-GlcNAcylation is generally believed to act as a metabolic integrator in numerous signalling pathways. The stoichiometry of this modification is tightly controlled throughout all stages of development, with both hypo/hyper O-GlcNAcylation resulting in broad defects. In this Primer, we discuss the role of O-GlcNAcylation in developmental processes from stem cell maintenance and differentiation to cell and tissue morphogenesis.

KEY WORDS: O-GlcNAcylation, Glycosylation, Polycomb group proteins, Stem cells, Differentiation

Introduction

Proteins can be post-translationally modified in several different ways, including phosphorylation, ubiquitylation and glycosylation (Khoury et al., 2011; Varki, 2017). O-linked β-N-acetylglucosaminylation (O-GlcNAcylation) is a mono-glycosylation that can occur on serine and threonine residues of nucleocytoplasmic proteins (Torres and Hart, 1984; Holt et al., 1987). Like several other forms of glycosylation, O-GlcNAcylation requires uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) as a substrate for the transfer of the sugar moiety onto proteins (Haltiwanger et al., 1990). UDP-GlcNAc is the product of the highly conserved hexosamine biosynthetic pathway (HBP) and its concentrations are generally believed to depend on the levels of various metabolites in the cell, crucially glucose (Fig. 1) (Marshall et al., 2004; Swamy et al., 2016; Chiaradonna et al., 2018), leading to hypotheses about the role of O-GlcNAcylation as a metabolic integrator. However, this may be a tissue-specific effect because recent research has demonstrated that elevated glucose has no effect on UDP-GlcNAc generation in *ex vivo* heart tissue (Olson et al., 2020). Mechanistically, O-GlcNAc transferase (OGT) is the enzyme that installs the sugar moiety on the target protein (Kreppel et al., 1997; Lubas et al., 1997) and the hydrolase O-GlcNAcase (OGA) catalyses its removal (Heckel et al., 1998; Gao et al., 2001). The presence of both an O-GlcNAc 'writer' and 'eraser' allows the levels of O-GlcNAcylation to rapidly change throughout development (Liu et al., 2012), as well as in response to changing UDP-GlcNAc concentration (Kreppel and Hart, 1999; Mariappa et al., 2011) and stress conditions, such as elevated temperature (Zachara et al., 2004; Radermacher et al., 2014; Mariappa et al., 2018). Modulation of O-GlcNAcylation is, in part, mediated by post-transcriptional and post-translational regulation of OGT and OGA, although the details of how these mechanisms interplay to control varying O-GlcNAcylation throughout development remain unknown (Whelan et al., 2008; Bullen et al., 2014; Tan et al., 2021). Conversely, an important function of O-GlcNAcylation is the regulation of other post-translational modifications (PTMs), such as ubiquitylation (Ruan et al., 2013; Chen et al., 2021) and phosphorylation (Hart et al., 2011; Bauer et al., 2015), occurring either on or near otherwise modified residues (Kamemura et al., 2002; White et al., 2020), or regulating PTM writers (Dias et al., 2009) and erasers (Liu et al., 2020).

This Primer summarises the current state of knowledge on the role of O-GlcNAcylation in development from loss-of-function studies in various species, highlighting roles in gene regulation and cell signalling. We also discuss emerging themes in this field, including the roles of O-GlcNAcylation in nervous system development and in integrating metabolism with signalling pathways and transcriptional regulation.

O-GlcNAc and loss-of-function studies

O-GlcNAc transferase

In most metazoans, a lack of functional OGT protein is developmentally lethal. This was first observed in *Drosophila*, in which zygotic homozygosity for amorphic alleles of the gene encoding OGT, *super sex combs* (*sxc*), results in lethality at the pupal stage of development (Ingham, 1984; Gambetta et al., 2009; Sinclair et al., 2009). However, in the absence of maternally contributed *sxc* gene product, lethality in *sxc*-null *Drosophila* occurs late in embryonic development or during early larval stages (Ingham, 1984; Gambetta and Müller, 2014). In vertebrates, the importance of OGT is more pronounced. Even in culture, mammalian cells cannot survive in the absence of *Ogt* and the loss of OGT protein is embryonically lethal in mice (Shafi et al., 2000; O'Donnell et al., 2004). Due to this lethality upon global loss of OGT, several groups have attempted to characterise the function of OGT in individual cell types using tissue-specific knockouts. While many conditional knockouts of *Ogt* result in lethality (Cheng et al., 2020; Xiong et al., 2022), this approach has demonstrated the far-ranging roles of the gene in development (Table 1). For example, the excision of *Ogt* in neuronal stem cells results in broad cortical malformations (Cheng et al., 2020), while conditional knockout of the gene in kidney podocytes results in defects in postnatal maturation of these cells (Ono et al., 2017).

It has been recently shown that several individuals with intellectual disability (ID) carry mutations in *OGT*, with family pedigree analysis convincingly indicating the causality of the mutations (Niranjan et al., 2015; Vaidyanathan et al., 2017; Willems et al., 2017; Pravata et al., 2019; Pravata, et al., 2020a). The disorder, termed OGT-linked congenital disorder of glycosylation (OGT-CDG), presents with a range of clinical signs beyond ID that are generally indicative of broad developmental defects, including clinodactyly (curved fingers), short stature and facial dysmorphisms (Pravata et al., 2020b). Several causal *OGT*

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Development (2023) 150, dev201370. doi:10.1242/dev.201370

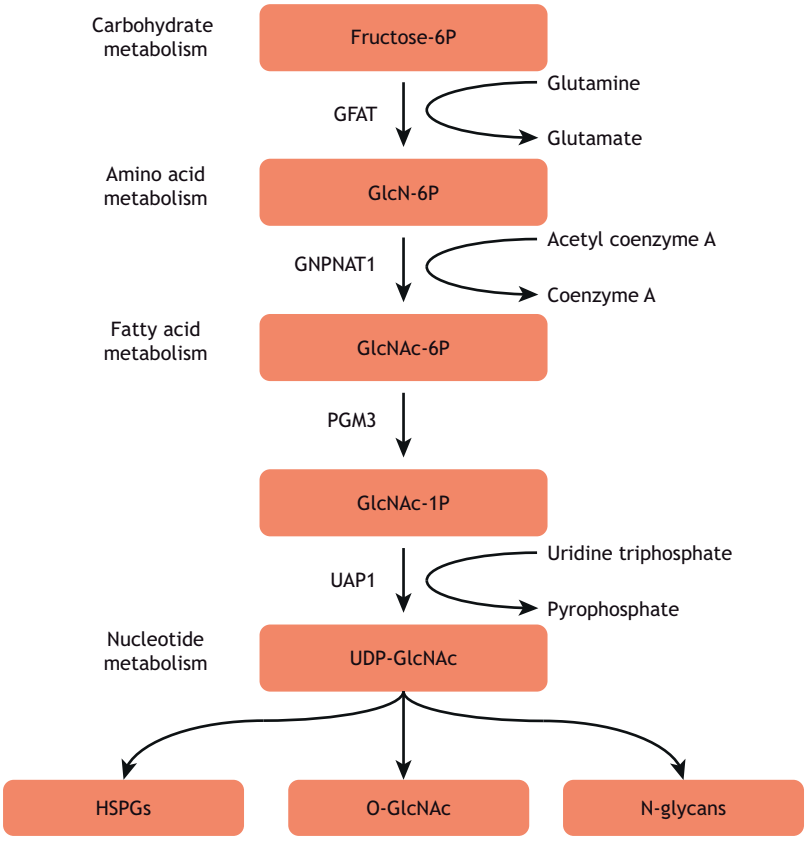

Fig. 1. The hexosamine biosynthetic pathway. The hexosamine biosynthetic pathway is responsible for the generation of UDP-GlcNAc and is highly conserved across metazoans. The first and rate-limiting enzyme of this pathway is glutamine fructose-6-phosphate amidotransferase (GFAT), followed by glucosamine 6-phosphate N-acetyltransferase (GNPNAT1), phosphoglucomutase 3 (PGM3) and UDP-N-acetylhexosamine pyrophosphorylase (UAP1). This pathway uses 2-3% of glucose (Marshall et al., 1991) in the cell and its product UDP-GlcNAc is a crucial substrate for several types of glycosylation, including O-GlcNAcylation, heparan sulphate proteoglycans (HSPGs) and N-glycans.

mutations in ID do not result in measurable decreases in global O-GlcNAcylation in patient-derived fibroblasts (Willems et al., 2017) or when modelled in mouse embryonic stem cells (mESCs) (Pravata et al., 2019), due to compensatory mechanisms, such as OGA downregulation.

O-GlcNAcase

In contrast to OGT deficiency, the lack of OGA appears to be better tolerated by most animals (Table 2). Flies lacking OGA are fully viable and fertile, presenting with only a mild phenotypes, such as larger adult size, a semi-penetrant oogenesis defect (Akan et al., 2016, 2021), mild neuronal and behavioural phenotypes (Muha et al., 2020), and increased intestinal stem cell proliferation (Na et al., 2020). Unlike in *Drosophila*, the lack of OGA or its catalytic activity in mice results in broad developmental defects in several organs and pre/perinatal lethality (Table 2) (Yang et al., 2012; Muha et al., 2021). Mice lacking OGA present with reduced body weight as early as embryonic day (E)14.5 (Yang et al., 2012), along with reduced lung alveolar space (Yang et al., 2012), reduced brain size along with a relative increase in ventricle size (Muha et al., 2021), as well as defects in glycogen storage and mobilisation, which are hypothesised to be key contributors to perinatal lethality in the absence of OGA function (Keembiyehetty et al., 2015).

These findings underscore the importance of maintaining appropriate O-GlcNAcylation throughout development, as even slight perturbations to O-GlcNAc homeostasis, as seen in OGT-CDG, can result in pronounced effects. With over 5000 proteins in the human proteome identified as O-GlcNAc modified, some of these modified multiple sites (Wulff-Fuentes et al., 2021); it remains a major challenge to identify functionally important sites and their role in developmental processes.

O-GlcNAcylation in embryonic stem cells

As mentioned above, O-GlcNAcylation is essential from the earliest stages of development and OGT is essential even in mESCs (Shafi et al., 2000). However, beyond being required for general cell viability, O-GlcNAcylation plays an important role in maintaining proliferation and pluripotency (Fig. 2). In mESCs, elevating O-GlcNAcylation by inhibiting OGA delays differentiation, while decreasing O-GlcNAcylation through knockdown of OGT impairs stem cell self-renewal (Jang et al., 2012; Speakman et al., 2014). The effects of O-GlcNAcylation on mESC maintenance appear to occur through the modulation of several pathways, including core pluripotency factors (such as Oct4 and Sox2), auxiliary factors [such as estrogen-related receptor β (ESRRB), phosphokinase PKCζ and proteasome activator subunit 3 (Psme3)] and epigenetic regulators (Jang et al., 2012; Shi et al., 2013; Miura et al., 2018; Hao et al., 2019; Pecori et al., 2021).

Pluripotency factor targets of O-GlcNAcylation

The effects of O-GlcNAcylation on protein function have largely been revealed by amino acid substitutions of serine/threonine residues that act as O-GlcNAc target sites. For example, alanine mutagenesis of O-GlcNAc sites in the DNA-binding domain of both mouse and human Oct4 (T228A and T235A, respectively) reduces Oct4 transcriptional activity in mouse embryonic fibroblasts and human cancer cells, respectively (Jang et al., 2012; Constable et al., 2017). Evidence that this effect is due to O-GlcNAcylation (and not to other post-translational modifications of Oct4 that occur in the same region) comes from the observation that OGT overexpression increases Oct4 transcriptional activity (Jang et al., 2012; Constable et al., 2017). Similarly, O-GlcNAcylation of serine 25 of ESRRB promotes pluripotency and self-renewal through increasing the stability and transcriptional activity of the protein

Development (2023) 150, dev201370. doi:10.1242/dev.201370

Table 1. Summary of O-GlcNAc transferase loss-of-function studies in which O-GlcNAc transferase is either completely absent or its catalytic activity is completely abolished

Species	Genotype (tissue)	Phenotype	Reference
Mouse	*αSMA-Cre, Ogt^{loxp}* (smooth muscle)	Postnatal lethality, dilated heart and heart failure	Xiong et al. (2022)
	DAT-Cre, Ogt^{loxp} (dopaminergic neurons)	Postnatal lethality, reduced weight/food intake and reduced activity	Shao et al. (2022)
	Cyp19-Cre, Ogt^{loxp} (placenta)	Increased number of β-islets with reduced size after a high-fat diet, reduced weight postnatally, increased corticosterone in males and reduced corticosterone in females	Howerton and Bale (2014); Moore et al. (2021)
	hGFAP-Cre, Ogt^{loxp} (neural stem cells)	Decreased body and brain weight, post-natal lethality, decreased cortical thickness, and impaired cerebellar development	Cheng et al. (2020); Chen et al. (2022)
	Troponin T- Cre, Ogt^{loxp} (cardiomyocytes)	Gross morphological heart defects (e.g. small atria and dilated ventricles)	Mu et al. (2020)
	Mlc1f-Cre, Ogt^{loxp} (skeletal muscle)	Reduced body mass on a high-fat diet	Murata et al. (2018)
	Nphs2-Cre, Ogt^{loxp} (kidney podocyte)	Impaired post-natal podocyte maturation and proteinuria	Ono et al. (2017)
	Syn1-Cre, Ogt^{loxp} (neuronal)	Partial embryonic lethality, reduced weight and abnormal locomotor activity	O'Donnell et al. (2004)
	Zp3-Cre, Ogt^{loxp} (female germ line)	Post-implantation embryonic lethality	Shafi et al. (2000); O'Donnell et al. (2004)
	αMHC-Cre, Ogt^{loxp} (cardiomyocytes)	Perinatal lethality, increased heart weight and increased ventricular volume	Watson et al. (2014)
Drosophila	*sxc^{1}* (global knockout)	Lethal homeotic transformations (e.g. ectopic sex combs and Hox gene expression)	Ingham (1984); Gambetta and Müller (2014)
	sxc^{K872M} (global catalytic inactivity)	Lethality	Mariappa et al. (2015)

(Hao et al., 2019). O-GlcNAcylation also directly modulates protein-protein interactions between Oct4 and ESSRB, as well as other regulators of stemness (Hao et al., 2019). However, in human cells, the effects of O-GlcNAcylation on Oct4 and ESRRB function have primarily been observed in cancer cells. Although additional research in human embryonic stem cells is required to understand the role of O-GlcNAcylation on these two proteins, the effects of O-GlcNAcylation on Oct4 transcriptional activity are not conserved in human embryonic stem cells, casting doubt on the importance of this mechanism in human development (Constable et al., 2017).

Whereas O-GlcNAcylation of Oct4 and ESRRB appears to promote stemness (Jang et al., 2012; Constable et al., 2017; Hao et al., 2019), Sox2 O-GlcNAcylation has mixed effects on maintaining pluripotency and self-renewal, depending on the site modified (Myers et al., 2016; Kim et al., 2021). Sox2 O-GlcNAcylation occurs in the transactivation domain and two neighbouring sites have been the focus of recent studies (Myers et al., 2016; Kim et al., 2021). Alanine mutagenesis of Sox2 threonine 258 increases the expression of early differentiation genes and reduces ectodermal marker genes in mESCs, indicating that threonine 258 O-GlcNAcylation is required for the function of Sox2 in self-renewal and regulation of the expression of ectoderm-lineage genes (Kim et al., 2021). Conversely, O-GlcNAcylation of Sox2 at serine 248 decreases its self-renewal activity and alanine mutagenesis of this site increases the reprogramming efficiency of somatic cells to induced pluripotent stem cells (Myers et al., 2016). O-GlcNAcylation of Sox2 at serine 248 impairs its interaction with poly ADP-ribose polymerase 1 (Parp1), a protein known to cooperatively regulate stemness, and thus Sox2 genomic binding (Lai et al., 2012). Given the antagonistic effects of O-GlcNAcylation of these two adjacent sites, the regulation of their modification and relative importance during development remains a pertinent question.

O-GlcNAcylation and epigenetics in pluripotency

In addition to directly controlling transcription factors involved in pluripotency, epigenetic markers required for stem cell maintenance are regulated by O-GlcNAcylation. In particular, DNA and histone methylation are sensitive to OGT activity because methylation modification enzymes and enzymes in the metabolic pathway that generates the most common methyl donor for methylation, S-adenosylmethionine (SAM), are regulated by O-GlcNAcylation (Fig. 2). Depletion of adenosylhomocysteinase (AHCY), a key enzyme in the SAM-generating methionine cycle, reduces the expression of pluripotency markers and increases the expression of differentiation markers through the loss of histone 3 lysine 4 (H3K4) methylation (Zhu et al., 2020). AHCY is O-GlcNAcylated on threonine 136, increasing its catalytic efficiency by almost an order

Table 2. Summary of O-GlcNAc transferase loss-of-function studies in which O-GlcNAc transferase is either completely absent or its catalytic activity is completely abolished

Species	Genotype	Phenotype	Reference
Mouse	*Oga* KO (global knockout)	Perinatal lethality, reduced embryo size and weight, decreased alveolar space, and decreased placental vascularisation	Keembiyehetty et al. (2015); Yang et al. (2015)
	Oga^{D285A} (global catalytic inactivity)	Perinatal lethality, reduced body weight and size, and brain ventricle enlargement	Muha et al. (2021)
	Nestin-Cre, Oga^{floxl-} (neural progenitor cells)	Gross brain abnormalities, such as reduced olfactory bulb and pituitary size, as well as enlarged ventricles. Altered neuronal differentiation.	Olivier-Van Stichelen et al. (2017)
Drosophila	*Oga^{KO}* (global knockout)	Increased body size, impaired locomotor and habituation function, and overgrowth at the neuromuscular junction	Muha et al. (2020); Akan et al. (2016, 2021)

Development (2023) 150, dev201370. doi:10.1242/dev.201370

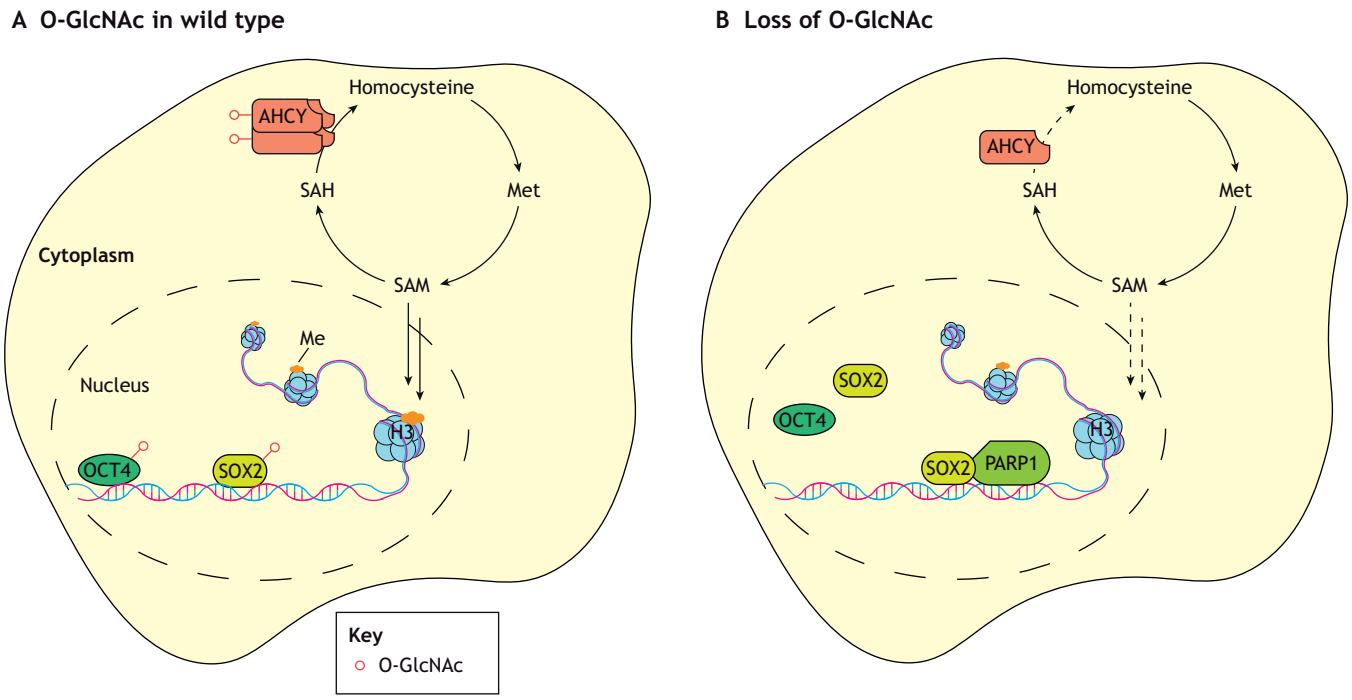

Fig. 2. Role of O-GlcNAcylation in embryonic stem cell maintenance. (A) Mouse embryonic stem cells (mESCs) with normal O-GlcNAcylation (O-GlcNAc) are maintained by a variety of transcriptional regulators. Some of the most important, the Yamanaka factors, can be used to induce pluripotency in terminally differentiated cells. Among these are OCT4 and SOX2, both of which are functionally O-GlcNAc modified. OCT4 transcriptional activity is regulated by O-GlcNAc in both its DNA-binding and transactivation domains, with increased O-GlcNAc promoting transcriptional activity. The effects of O-GlcNAc of SOX2 are more site specific: although both occur in the transactivation domain, SOX2 O-GlcNAcylation can either increase its transcriptional activity or change its DNA occupancy by altering its interactions with other proteins important in maintenance of stemness. Histone (H3) methylation (Me) is also important for perpetuating stemness. A crucial metabolic pathway for maintaining appropriate levels of methylation is the methionine (Met) cycle that produces S-adenosyl methionine (SAM), a donor substrate for methyltransferases. O-GlcNAc plays a crucial role in this cycle by enhancing the activity of adenosylhomocysteinase (AHCY), which converts S-adenosylhomocysteine to homocysteine and adenosine. (B) Loss of O-GlcNAcylation disrupts transcriptional regulation, e.g. by changing SOX2 protein interactions with co-factors such as poly [ADP-ribose] polymerase 1 (PARP1), which results in differentiation.

of magnitude through facilitating AHCY homotetramerization and thereby promoting stem cell maintenance (Zhu et al., 2020). O-GlcNAcylation also regulates the function of histone methyltransferases directly (Chu et al., 2014; Lo et al., 2018; Parween et al., 2022), although this function has been investigated more extensively in the context of differentiation and so is discussed in greater detail later.

In the context of the role of DNA methylation in stem cell maintenance, the interplay between the TET (Ten-eleven translocation) family of proteins and OGT has been a focus of research. TET proteins (TET1-TET3) are key enzymes responsible for demethylating DNA through converting 5-methylcytosine (5mC) to 5-hydroxymethylcytosine and in so doing regulate gene expression (Tahiliani et al., 2009). TET1 and TET2 are particularly enriched in mESCs, and a loss of protein function results in earlier differentiation (Ito et al., 2010; Shi et al., 2013). The loss of self-renewal and increased differentiation upon knockdown of TET1 and/or TET2 is likely due to their role in derepression (by hydroxylating 5mC) of core pluripotency factors, primarily Nanog (Ito et al., 2010). One of the first functions ascribed to OGT/TET interactions was the stabilising effect of O-GlcNAcylation on TET1 (Shi et al., 2013); however, more recent research shows a more-complex relationship between OGT and the TET family of proteins. TET1 activity is regulated by its interaction with OGT, with OGT binding-deficient TET1 unable to rescue defects in hematopoietic stem cell production in TET knockout zebrafish (Hrit et al., 2018). The mechanistic details of how OGT and O-GlcNAcylation affect stability and activity of TET proteins remain to be fully understood, although extensive crosstalk between O-GlcNAcylation and phosphorylation occurs on TET proteins (Bauer et al., 2015). TET proteins have also been found to regulate OGT activity, promoting O-GlcNAcylation of chromatin-associated protein complexes and histones (Chen et al., 2013; Deplus et al., 2013). For example, both TET2 and TET3 promote O-GlcNAcylation of host cell factor 1 (HCF1), which regulates the integrity of the H3K4 methyltransferase complex SET1/COMPASS, as well as its binding to chromatin (Deplus et al., 2013). Although the consequences of this interaction on stem cell maintenance have not been investigated, SET1 is known to promote mESC colony formation, potentially through its interaction with Oct4, enhancing its activity (Fang et al., 2016).

It is important to note that, despite the high degree of conservation of O-GlcNAc sites on the pluripotency factors mentioned here, the phenotypic effects of perturbations in O-GlcNAc addition and removal on pluripotency maintenance in human pluripotent stem cells are milder relative to mouse cells (Maury et al., 2013; Andres et al., 2017). Human embryonic and induced pluripotent stem cells are unperturbed by OGA or OGT manipulation, although increasing or decreasing O-GlcNAcylation does accelerate their rate of differentiation (Maury et al., 2013; Andres et al., 2017). Whether this is a result of broader cellular differences between mouse and human pluripotent stem cells (Schnerch et al., 2010) or of differences in the role of O-GlcNAcylation on target proteins remains to be fully explored.

Development (2023) 150, dev201370. doi:10.1242/dev.201370

O-GlcNAcylation in differentiation and cell fate determination

The first function ascribed to OGT (before its molecular characterisation) was its role as a polycomb group (PcG) protein (Ingham, 1984). More recently, the diverse roles of O-GlcNAcylation in differentiation and cell fate determination have become more extensively investigated. The importance of O-GlcNAcylation in neuronal development is particularly pronounced; however, many unanswered questions remain regarding mechanisms regulated by this PTM, across different germ layers.

OGT as a polycomb group gene

Proteins encoded by PcG genes are important transcriptional regulators that prevent ectopic gene expression during development. Notable targets of PcG silencing are the homeotic Hox genes, making PcG proteins central to the maintenance of cell identity in the anterior-posterior axis (Struhl and Akam, 1985; Golbabapour et al., 2013). PcG proteins assemble into histone-modifying complexes that act in concert to maintain gene repression at loci adjacent to genomic regions called polycomb response elements (PREs) (Muller and Bienz, 1991; Horard et al., 2000; Kassis and Brown, 2013). In *Drosophila*, O-GlcNAcylation prevents the aggregation of a stoichiometric component of polycomb repressive complex 1 (PRC1), Polyhomeotic (Ph) (Fig. 3) (Gambetta and Müller, 2014). In the absence of OGT activity, or the serine/threonine-rich domain of Ph that is O-GlcNAc modified, Ph forms aggregates that prevent PRC1-mediated maintenance of Hox gene repression, such as *ultrabithorax* (Ubx) (Gambetta and Müller, 2014). Although it has not been investigated whether this observation translates to vertebrates, human orthologs of Ph (PHC2 and PHC3) also aggregate *in vitro* in the absence of O-GlcNAcylation (Gambetta and Müller, 2014). In human embryonic stem cells, the catalytic subunit of PRC1 (RING1B) is also modified by O-GlcNAc, altering its chromosomal distribution (Maury et al., 2015): O-GlcNAcylated RING1B is enriched at genes implicated in neuronal differentiation and development, relative to the unmodified protein (Maury et al., 2015). O-GlcNAcylation decreases upon neuronal differentiation of human ESCs (Maury et al., 2013); therefore, the loss of RING1B O-GlcNAcylation may be important for the derepression of genes involved in neuronal differentiation.

In addition to PRC1, early genetic interaction studies proposed a role for OGT, and by extension O-GlcNAcylation, in the function of other PcG complexes. In *Drosophila, sxc* amorphic alleles enhance homeotic transformations as a result of mutations in elements of either the polycomb repressive complex 2 (PRC2) or the polycomb repressive-deubiquitylase complex (PR-DUB) (Campbell et al., 1995). Recent research has demonstrated the molecular functions of O-GlcNAcylation in these complexes in mammalian systems. The primary focus has been on the histone methyltransferase subunit enhancer of zeste homologue 2 (EZH2) of PRC2 (Fig. 3). *In vitro*, O-GlcNAcylation increases EZH2 stability, specifically when this protein is not in complex with other PRC2 components, and enhances its histone methyltransferase activity (Chu et al., 2014; Lo et al., 2018). However, *in vivo* research indicates that elevated O-GlcNAcylation can also negatively regulate EZH2 function. In a rat model of maternal hyperglycaemia, a condition leading to elevated embryonic O-GlcNAcylation, EZH2 has increased phosphorylation at threonine 311 (Parween et al., 2022). Phosphorylation at this site hinders the association between EZH2 and SUZ12, another PRC2 component (Wan et al., 2018). The consequent deregulation of PRC2 activity results in misexpression of neurogenic genes, causing elevated neuronal marker expression at earlier stages of development, a phenotype that can be reversed by OGT inhibition (Parween et al., 2022). However, how O-GlcNAcylation positively regulates phosphorylation of EZH2 remains unknown. Threonine 311 of EZH2 is phosphorylated by AMP-activated protein kinase (AMPK) (Wan et al., 2018), which is likely more active when O-GlcNAcylated in specific contexts (Bullen et al., 2014; Xu et al., 2014). However, it remains unknown whether this mechanism is responsible for the phenotypes seen in hyperglycaemic mouse embryos, or whether other mechanisms of O-GlcNAc/phosphorylation crosstalk are involved. The effects of

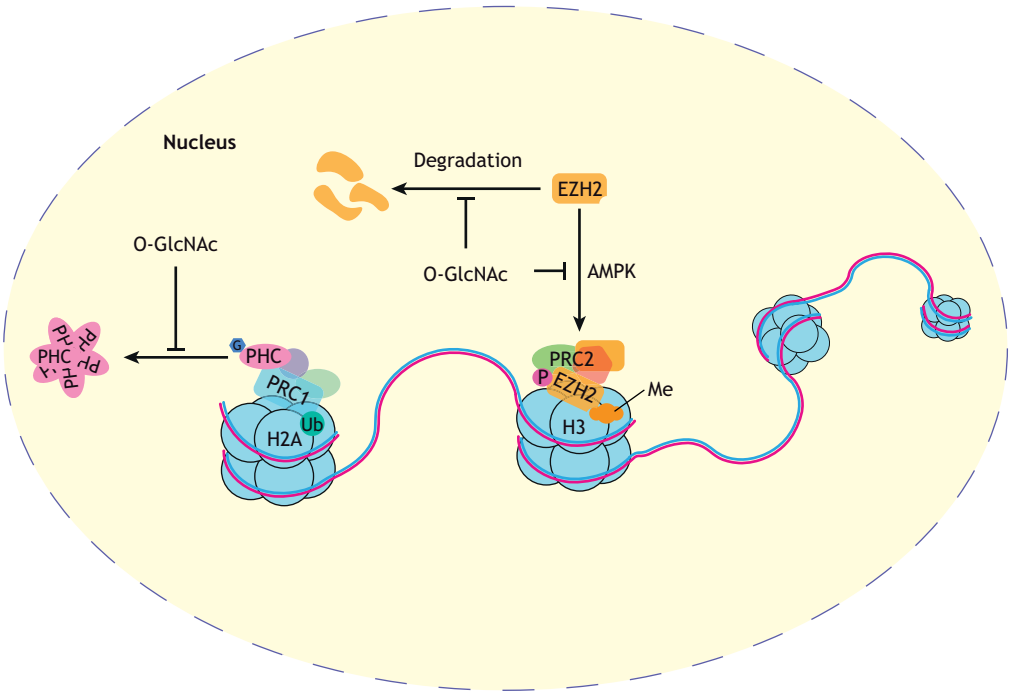

Fig. 3. O-GlcNAcylation in PcG protein function. O-GlcNAcylation (O-GlcNAc) is important for the functioning of several PcG proteins; notably, preventing the aggregation of polyhomeotic (PHC), a component of polycomb repressive complex 1 (PRC1), which catalyses the ubiquitylation (Ub) of histone 2A (H2A). In histone 3 (H3) methylating (Me) polycomb repressive complex 2 (PRC2), O-GlcNAc appears to play an important role in preventing the incorporation of enhancer of zeste homolog 2 (EZH2) into the complex by preventing its phosphorylation by AMP-activated protein kinase (AMPK). However, in addition to this role, when not in complex with other components of PRC2, O-GlcNAc prevents the degradation of EZH2. G, O-GlcNAc; P, phosphorylation.

Development (2023) 150, dev201370. doi:10.1242/dev.201370

O-GlcNAcylation on EZH2 may also, in part, explain why human iPSCs acquire neuronal markers earlier in differentiation in the presence of OGA inhibitors (Parween et al., 2017).

The component of the PR-DUB that genetically interacts with *sxc* is Additional sex combs (*Asx*) (Milne et al., 1999). *Asx* is important for the functioning of both PcG and the opposing trithorax group (TrxG) proteins, and, therefore, plays a role in both restricting and promoting the expression of Hox genes (Milne et al., 1999; Li et al., 2017). All the human orthologs of *Asx* are O-GlcNAcylated (Wulff-Fuentes et al., 2021), with O-GlcNAcylation stabilising the ortholog Asx-like 1 (ASXL1) (Inoue et al., 2018). The functional significance of the relationship between OGT and ASXL1 remains understudied; however, some evidence indicates that reduced O-GlcNAcylation of ASXL1 results in deficient myeloid differentiation by inhibiting H3K4 methylation (Inoue et al., 2018).

O-GlcNAc in cell fate determination

Several studies have found that the precise regulation of O-GlcNAcylation is required for cells to differentiate into a specific fate. Several cell types, such as cardiomyocytes (Kim et al., 2009), myocytes (Ogawa et al., 2012), keratinocytes (Sohn et al., 2014), neutrophils and erythroid cells (Zhang et al., 2019), require reduced levels of O-GlcNAcylation to acquire their cell fate, while several others, such as osteoblasts (Koyama and Kamemura, 2015), adipocytes (Ishihara et al., 2010) and chondrocytes (Andrés-Bergós et al., 2012), require increased levels of O-GlcNAcylation to differentiate. In some cases, the inability of cells to acquire a specific identity upon O-GlcNAc deregulation may simply reflect the role of O-GlcNAcylation in maintaining stemness. However, important roles for O-GlcNAcylation in the function of cell fate-determining transcription factors have also been identified, such as Sp1 (Yang et al., 2001; Sohn et al., 2014). In keratinocytes, upon differentiation, global levels of O-GlcNAcylation decrease, including O-GlcNAcylation of Sp1. This results in a de-repression of Sp1 transcriptional activity, which increases the expression of genes encoding structural proteins important for the formation of the protective outer layer of the epidermis: the stratum corneum (Sohn et al., 2014).

O-GlcNAc and neural development

The role of O-GlcNAcylation in neuronal differentiation has been of particular interest in recent years due to its relevance to OGT-CDG. An increase in mESC O-GlcNAcylation impairs differentiation towards a neuronal cell fate and mice lacking *Oga* in neuronal precursor cells present with increased numbers of immature neurons (Olivier-Van Stichelen et al., 2017). Conversely, the loss of O-GlcNAcylation in neural stem cells (NSCs) through genetic ablation of *OGT* using Cre-Lox recombination or knockdown with short hairpin RNA (shRNA) results in a depletion of these cells and impairs neurogenesis (Cheng et al., 2020; White et al., 2020; Chen et al., 2021; Shen et al., 2021), although reports of mechanisms causing this phenotype are conflicting. One explanation for impaired neurogenesis in the absence of OGT is via STAT3, a key transcription factor that promotes a glial cell fate (Cao et al., 2010; Hong and Song, 2014). O-GlcNAcylation of STAT3 prevents its phosphorylation on a nearby residue, thereby inhibiting its transcriptional activity (White et al., 2020). Although this effect has primarily been investigated in the context of ageing, it also extends to NSCs isolated from postnatal day (P) 1 mice. More recent research challenges the notion that O-GlcNAcylation impairs differentiation towards a neural fate and suggests that impaired neurogenesis occurs primarily because of NSC depletion. Specifically, shRNA knockdown of OGT in mouse embryos results in the preferential acquisition of a neuronal cell fate by transfected cells, an effect that can also be observed in human forebrain organoids (Shen et al., 2021). This shift towards a neuronal cell fate is accompanied by a reduction in the number of proliferating NSCs, explaining the reduced total number of differentiated neurons upon loss of OGT. Further supporting this mechanism of impaired neurogenesis, loss of OGT in mouse adult NSCs reduces proliferation and results in increased differentiation towards a neuronal cell fate, at the expense of glial differentiation (Chen et al., 2021). Mechanistically, this shift towards a neuronal cell fate can be explained by reduced levels of either β-catenin or reduced Notch signalling (Chen et al., 2021; Shen et al., 2021). Disentangling how such mechanisms may interact in the developing brain is crucial for the understanding conditions such as OGT-CDG or hyperglycaemia-induced neural tube defects (Kim et al., 2017).

O-GlcNAcylation in cell and tissue morphogenesis

Beyond controlling stem cell proliferation, differentiation and cell identity, OGT activity can also modulate morphogenesis. For example, in mouse embryos from hyperglycaemic dams, defects in neural tube closure can be reversed by OGT inhibition (Kim et al., 2017). In zebrafish, *ogt* overexpression disrupts epiboly and microtubule formation, resulting in thicker shorter microtubules (Webster et al., 2009). However, the mechanistic roles of O-GlcNAcylation in these phenotypes has not been further investigated.

Signalling pathways

More-detailed insights into the role of O-GlcNAcylation in morphogenesis have been gained from research in *Drosophila*, from genetic disruption of components of the HBP. For example, loss of *nesthocker* (*nst*), the *Drosophila* ortholog of phosphoglucomutase 3 (*PGM3*), results in defective cell migration of mesodermal cells during gastrulation and later defective tracheal formation (Mariappa et al., 2011). These defects are a result of altered fibroblast growth factor (FGF) signalling, wherein a crucial adaptor protein, Downstream of FGFR (Dof), requires O-GlcNAcylation for appropriate function. This is illustrated by phenotypic rescue of *nst* mutants by overexpression of a chimeric receptor retaining the FGF receptor extracellular domain while the intracellular domain is substituted for a tyrosine kinase that is not dependent on Dof for signal transduction. Although mechanistic details of the role of O-GlcNAcylation on Dof are not understood, the lack of additional research in this area is likely due to Dof not being conserved in mammals (Mariappa et al., 2011).

In addition to FGF signalling, O-GlcNAc is also linked to bone morphogenetic protein (BMP) signalling. In *Drosophila*, excessive activity of the BMP ligand decapentaplegic (Dpp) manifests most visibly as a tissue morphogenesis phenotype, with dorsal puckering from excess dorsal movement of epithelial cells. This phenotype is also observed in *sxc* mutant embryos and when the HBP is disrupted, e.g. in *Drosophila* that are homozygous for a hypomorphic allele of the gene *Mummy* (*mmy*) (Moulton et al., 2020). *mmy* is the ortholog of the human gene UDP-N-acetylglucosamine pyrophosphorylase (*UAP1*) (Humphreys et al., 2013), which encodes an enzyme that catalyses the formation of UDP-GlcNAc from N-acetylglucosamine-1-phosphate and UTP – the final step in the HBP (Mio et al., 1998) (Fig. 1). Although proteomics approaches have identified that some elements of the Dpp transcriptional activator complex (AP-1) are O-GlcNAc

modified (Selvan et al., 2017), the phenotypes seen in *mmy* mutants are more likely conveyed through defective O-GlcNAcylation of the type I BMP receptor saxophone (Sax) (Moulton et al., 2020). In the absence of functional OGT, the *Drosophila* SMAD ortholog, Mothers against dpp (Mad), is ectopically phosphorylated by Sax, causing the dorsalising phenotypes seen in *mmy* and *sxc* mutant embryos. Interestingly, embryos with wild-type *sxc* from mothers fed a sugar-free diet present with a similar phenotype to embryos homozygous for an amorphic allele of *sxc*, demonstrating that the role of O-GlcNAcylation in conveying metabolic signals in development is conserved from *Drosophila* to mammals (Moulton et al., 2020). Whether this particular mechanism is conserved in vertebrates remains to be investigated, although the putatively modified region of Sax is conserved in its human orthologs (Moulton et al., 2020).

Neurite morphology

Another understudied function of O-GlcNAcylation is its role in neurite outgrowth and axonal branching. OGA overexpression in chicken embryonic forebrain neurons is associated with increased primary axon length and the number of neurons entering a branching program (Francisco et al., 2009), whereas knockout of OGT in mouse embryonic cortical neurons reduces their total axonal length (Cheng et al., 2020). The mechanism by which loss of O-GlcNAcylation promotes axon branching is hypothesised to be downstream of protein kinase A (PKA) because PKA activation increases axon branching and this is prevented when OGA is inhibited (Francisco et al., 2009); however, specific conveyors have not been further investigated. O-GlcNAcylation also affects dendrite outgrowth in cultured hippocampal and cortical neurons (Cheng et al., 2020; Shen et al., 2021), with neurite outgrowth of the former rescued by overexpression of β-catenin (Shen et al., 2021), which is reduced in cells lacking OGT. β-Catenin is stabilised by O-GlcNAcylation (Olivier-Van Stichelen et al., 2014); therefore, it is likely that the effects of reduced O-GlcNAcylation on neurite outgrowth are directly mediated by this role of OGT. Similar defects have been observed in models of OGT-CDG. In *Drosophila*, both loss of O-GlcNAcase and mutations in *sxc* that are analogous to those seen in individuals with ID result in disrupted neuromuscular junction formation (Muha et al., 2020; Fenckova et al., 2022), while neurons differentiated from mESCs carrying a patient mutation are characterised by reduced neurite outgrowth (Pravata et al., 2019).

Concluding remarks

In summary, O-GlcNAcylation exerts broad effects on development by modulating a variety of cellular processes, including transcriptional regulation, epigenetics and metabolic pathways. The role of O-GlcNAcylation in these processes is diverse, although key among them is the crosstalk of O-GlcNAcylation with other PTMs. An emerging theme in the role of O-GlcNAc in development is its connection to metabolic states, in part mediated through the HBP (Mariappa et al., 2011; Humphreys et al., 2013; Moulton et al., 2020; Wong et al., 2020). In humans, defective maternal metabolic homeostasis, such as poorly managed pregestational diabetes mellitus, can also affect development (Ornoy et al., 2015). Interestingly, much like mutations in *OGT*, some of the most pronounced clinical manifestations of maternal pregestational diabetes mellitus during development affect the central nervous system, including neural tube defects and autism (Ornoy et al., 2015). Initial studies have shown that maternal hyperglycaemia can negatively impact embryonic development, both through altering embryonic (Kim et al., 2017) and placental O-GlcNAcylation (Dela Justina et al., 2017, 2018). The study of O-GlcNAcylation in development may lead to crucial discoveries related to the effects of metabolic states on development, particularly relevant in the light of an increase in individuals affected by metabolic disorders (Saklayen, 2018).

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