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SHORT REPORT

RING1B O-GlcNAcylation regulates gene targeting of polycomb repressive complex 1 in human embryonic stem cells

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Abstract
O-linked-N-acetylglucosamine (O-GlcNAc) post-translationally modifies and regulates thousands of proteins involved in various cellular mechanisms. Recently, O-GlcNAc has been linked to human embryonic stem cells (hESC) differentiation, however the identity and function of O-GlcNAc proteins regulating hESC remain unknown. Here, we firstly identified O-GlcNAc modified human stem cell regulators such as hnRNP K, HP1γ, and especially RING1B/RNF2. Thereafter, we focused our work on RING1B which is the catalytic subunit of the polycomb repressive complex 1 (PRC1) a major epigenetic repressor essential for pluripotency maintenance and differentiation. By point-mutation, we show that T^{250}/S^{251} and S^{278} RING1B residues are bearing O-GlcNAc, and that T^{250}/S^{251} O-GlcNAcylation decreases during differentiation. O-GlcNAc seems to regulate RING1B-DNA binding as suggested by our ChIP-sequencing results. Non-O-GlcNAcylated RING1B is found to be enriched near cell cycle genes whereas O-GlcNAcylated RING1B seems preferentially enriched near neuronal genes. Our data suggest that during hESC differentiation, the decrease of RING1B O-GlcNAcylation might enable PRC1 to switch its target to induce neuron

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differentiation. Overall, we demonstrate that O-GlcNAc modifies and regulates an essential epigenetic tool, RING1B, which may contribute to hESC pluripotency maintenance and differentiation.

Introduction

O-linked N-acetylglucosamine (O-GlcNAc) post-translationally modifies specific serine and threonine residues from nucleocytoplasmic proteins (Torres and Hart, 1984). O-GlcNAc is dynamically added and removed by the O-GlcNAc transferase and hydrolase respectively to regulate protein–protein interaction, protein–DNA interaction, and protein stability (Ozcan et al., 2010). Even though thousands of nucleocytoplasmic proteins have already been reported (Hart and Akimoto, 2009), only few studies have recently started to establish a link between O-GlcNAcylation and stem cell pluripotency. O-GlcNAc transferase was shown to be essential for mouse embryonic stem cell (mESC) viability (Shafi et al., 2000) and for zebrafish proper morphogenesis (Webster et al., 2009). In mESC, cell pluripotency maintenance and somatic cell reprogramming is regulated by Oct4 O-GlcNAcylation (a pluripotency transcription factor) (Jang et al., 2012). In addition, mESC cardiac-differentiation was also shown to be controlled by global levels of O-GlcNAc (Kim et al., 2009). Moreover, we demonstrated that O-GlcNAc excess increased adipose commitment and decreased ectoderm commitment of differentiating hESC suggesting that O-GlcNAcylated proteins are regulating specific hESC differentiation pathways (Maury et al., 2013).

Polycomb repressive complex 1 (PRC1) is an epigenetic repressor essential for regulating pluripotency maintenance and differentiation (Rajasekhar and Begemann, 2007). PRC1 catalytic activity subunit, RING1B/RNF2 protein, monoubiquitinylates histone 2A (H2AK119ub) which in turn repress the expression of genes targeted by PRC1 (Vidal, 2009). Interestingly, Gambetta et al., 2009 showed in *Drosophila* embryos that PRC1 Ring (human RINGs ortholog) was enriched through its O-GlcNAc modification (supporting material, Fig. S5 Gambetta et al., 2009); suggesting that human RING1B protein might be O-GlcNAcylated. Interestingly, Speakman C., et al. also reported recently that some polycomb-target genes in

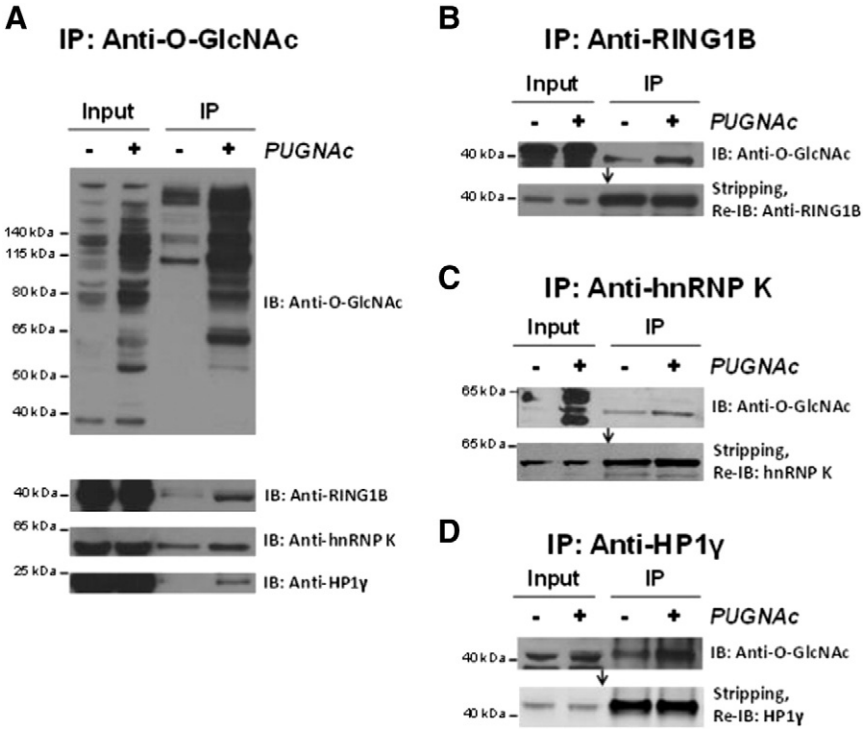


Figure 1 hnRNP K, HP1γ, and RING1B are O-GlcNAcylated in hESC. (A–D) Western and Eastern blot analyses of immunoprecipitated (IP) O-GlcNAcylated proteins; hnRNP K; HP1γ, and RING1B proteins from hESC treated without (−) or with (+) 100 μM of PUGNAc for 24 h. Of note, in (D) input and IP solutions were run under non-reducing conditions because HP1γ and antibody light chain co-elute and are difficult to distinguish under reducing conditions. Thus, HP1γ apparent molecular weight is changed from 25 kDa (reducing condition) to 40 kDa (non-reducing condition). CTD110.6 antibody was used for the immunoprecipitation and Eastern blots of O-GlcNAc.

mESC had an altered expression following O-GlcNAc excess (Speakman et al., 2014), suggesting that O-GlcNAc might directly or indirectly regulate the function of polycomb proteins.

Here, we started by investigating RING1B O-GlcNAcylation in hESC. Through various strategies (immunoprecipitation, point-mutations and mass spectrometry), we demonstrated that RING1B is O-GlcNAcylated on specific residues T^{250}/S^{251} and S^{278} and that RING1B O-GlcNAcylation decrease during hESC differentiation. Finally, chromatin immunoprecipitation experiment suggests that O-GlcNAcylation of RING1B might regulate PRC1 DNA-binding.

Materials and methods

Information related to Material and methods can be found in the Supplemental information section.

Results

RING1B is O-GlcNAcylated in hESC

We started by investigating the O-GlcNAcylation of RING1B by immunoprecipitation in hESC. As a positive control, we also tested two other stem cell regulators previously reported as being O-GlcNAcylated: hnRNP K and HP1γ (Drougat et al., 2012; Gambetta et al., 2009). hnRNP K is a RNA-binding protein regulating neuronal (Cao et al., 2012) and erythroid differentiations (Naarmann-de Vries et al., 2013). HP1γ is a heterochromatin-associated protein involved in gene repression and differentiation (Morikawa et al., 2013).

In our experiments, hnRNP K, HP1γ, and RING1B were all detected after IP of O-GlcNAcylated proteins (Fig. 1A). In addition, a higher amount of these proteins was immunoprecipitated from cells treated with PUGNAc (O-GlcNAc

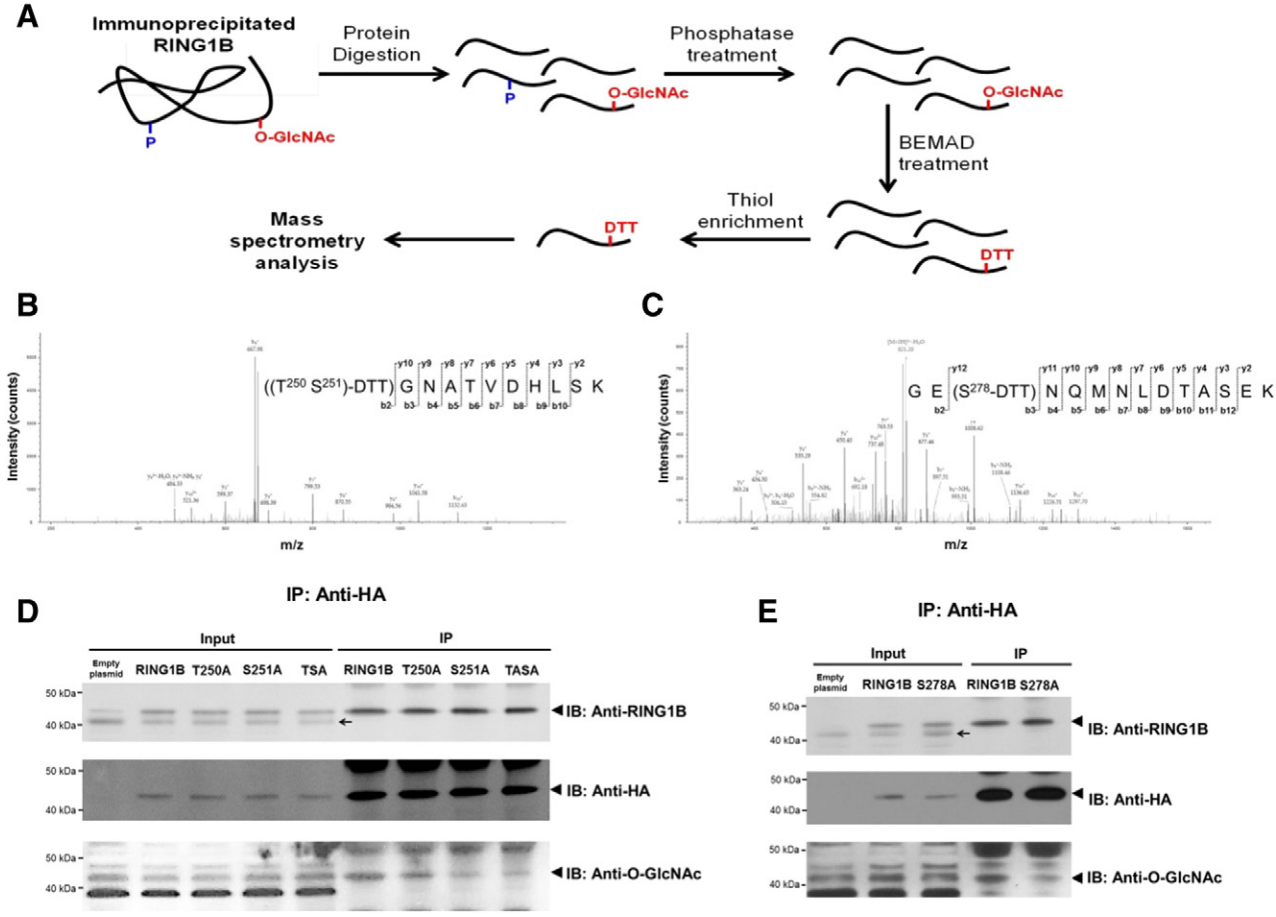


Figure 2 RING1B is O-GlcNAcylated at S^{250}/T^{251} and S^{278} and RING1B O-GlcNAcylation decreases during hESC differentiation. (A) Overview of RING1B treatment before mass spectrometry analysis. Briefly, immunoprecipitated RING1B protein was digested and subject to phosphatase treatment to remove phosphate groups. Then the peptides were subjected to BEMAD reaction in order to substitute O-GlcNAc group by DTT. Finally, the DTT-modified peptides were enriched on thiopropyl sepharose and analyzed by MS. (B–C) MS^2 analysis of RING1B DTT-modified peptides [(TS)-OGlcNAc]GNATVDHLSK and GE[S-DTT]NQMNLDTASEK by MS. (D–E) Western and Eastern blots analyses of hESC transfected with empty plasmid used as a negative control, HA-RING1B (noted RING1B) and mutated versions of HA-RING1B where some residues are substituted by alanine: residue T^{250} (noted T250A), residue S^{251} (noted S251A), residue S^{278} (noted S287A) and the double mutant T^{250} and S^{251} (noted TASA). Arrowheads point out the recombinantly expressed HA-RING1B proteins whereas arrows with tail point out the native RING1B protein.

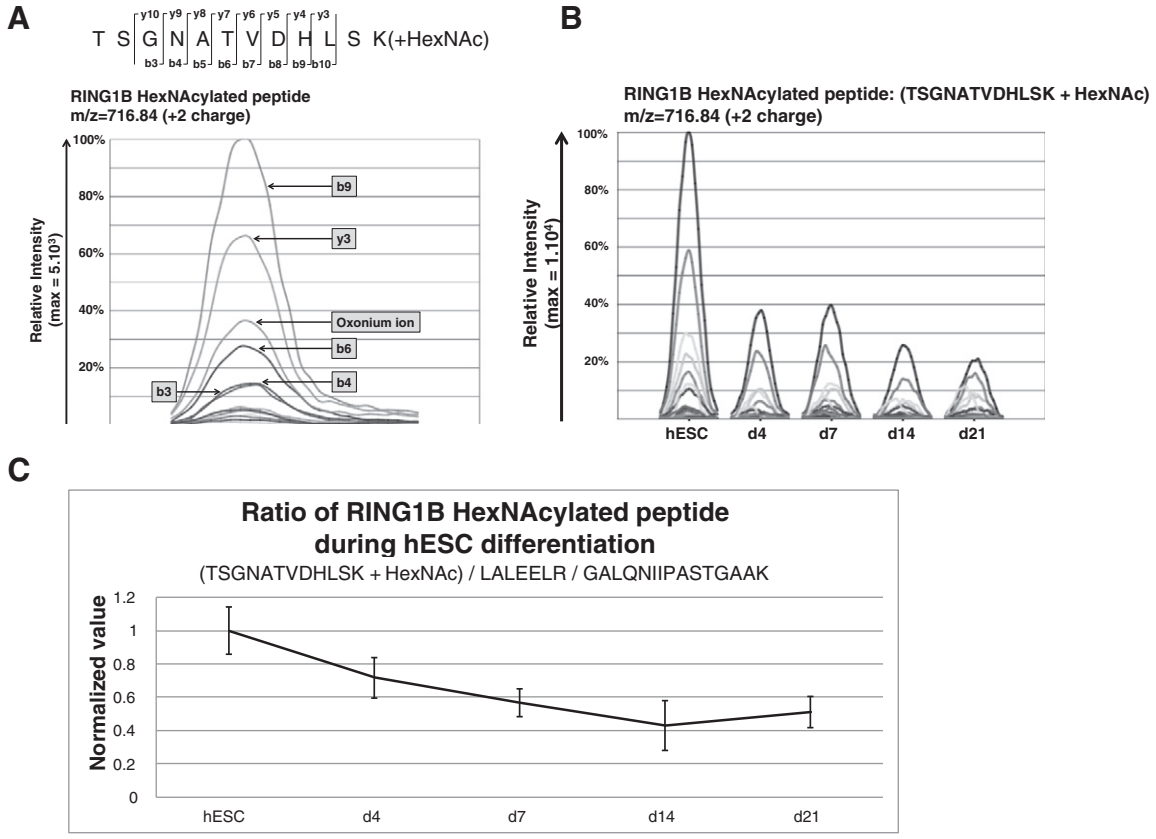


Figure 3 RING1B HexNAcylation decreases during hESC differentiation. (A) Representative LC-MRM-MS trace of RING1B HexNAcylated peptide (TSGNATVDHLSK + HexNAc). (B) Comparison of the LC-MRM-MS traces of RING1B HexNAcylated peptide (TSGNATVDHLSK + HexNAc) during hESC differentiation at d4, d7, d14, and d21 post-differentiation. (C) Comparison during hESC differentiation of the area values from the integration of (TSGNATVDHLSK + HexNAc) traces normalized by the area value of the non-modified RING1B peptide (Fig. S3D) and GAPDH peptide (Fig. S3E).

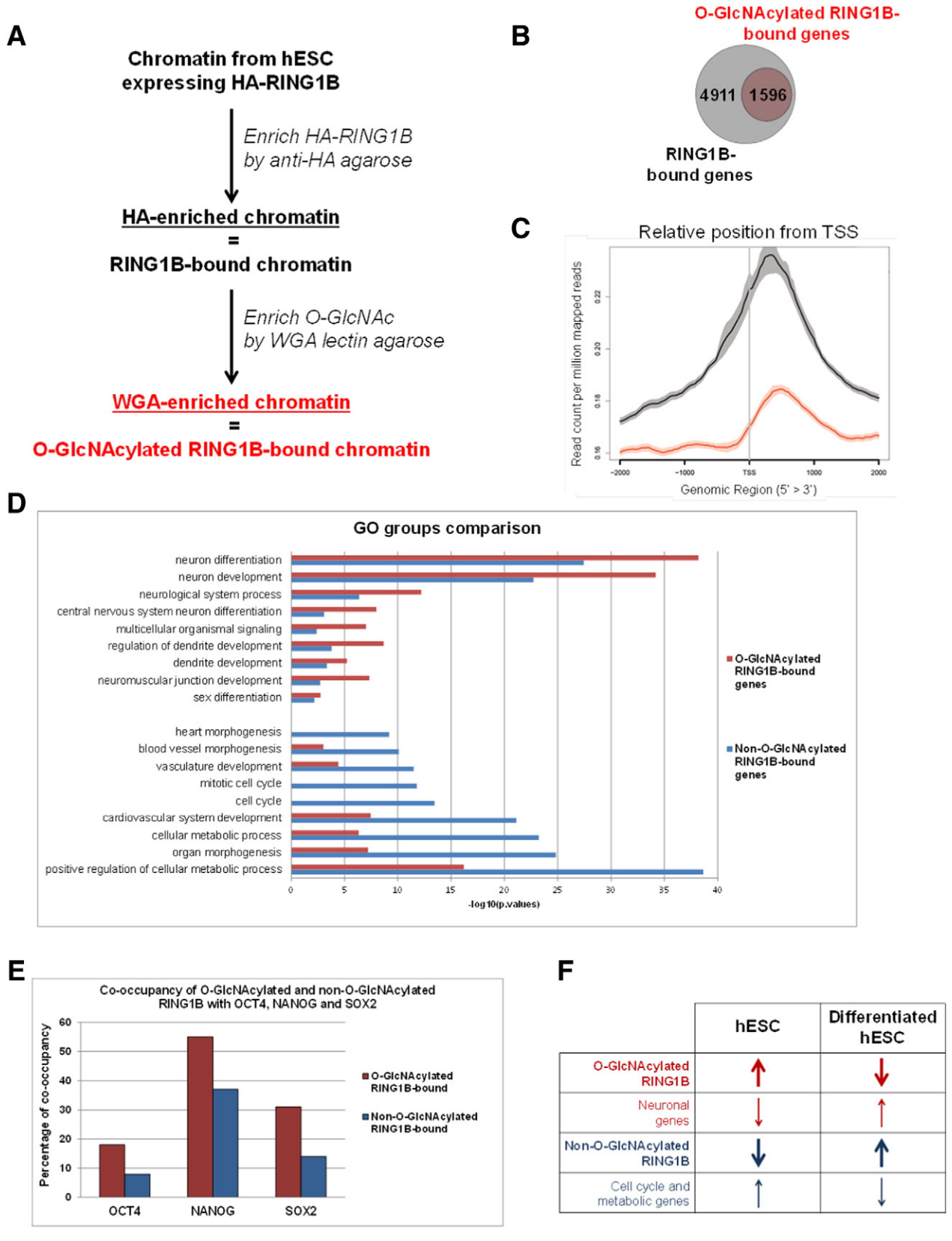
hydrolase inhibitor). This is consistent with the conclusion that these proteins are O-GlcNAcylated (Fig. 1A). These data were also confirmed by reverse IP of hnRNP K, HP1γ, and RING1B (Figs. 1B–D).

Localizing RING1B O-GlcNAcylation sites

To pinpoint RING1B O-GlcNAcylation sites, we used the BEMAD reaction to substitute O-GlcNAc (not stable through MS) by DTT (stable through MS) followed by a mass spectrometry (MS) analysis (Fig. 2A) (Wells et al., 2002). Following this strategy, two RING1B DTT-modified peptides were detected: [($T^{250}S^{251}$)-DTT]GNATVDHLSK (Fig. 2B); and GE[S^{278}-DTT]NQMNLDTASEK (Fig. 2C). Even though [($T^{250}S^{251}$)-DTT]GNATVDHLSK peptide was identified with a spectrum containing most of the fragment ions, this spectrum doesn't allow to precisely pinpoint the DTT modification between either T^{250} or S^{251}. To further validate these results, we recombinantly expressed mutated versions of HA-tagged RING1B in hESC. Following IP of the recombinant HA-RING1B, we show that the simple mutation of T^{250} (T250A), S^{251} (S251A) as well as the double mutation (TASA) into alanine all reduced HA-RING1B O-GlcNAcylation (Fig. 2D). These data suggest that both T^{250} and S^{251} residues are necessary for proper RING1B O-GlcNAcylation. Similarly, S^{278} simple mutation (S278A) reduced HA-RING1B O-GlcNAcylation (Fig. 2E). Then, it can be concluded that S^{278} residue is O-GlcNAc modified and that T^{250} and S^{251} are both required for proper O-GlcNAcylation of RING1B thus assuming that one of the two residues (either T^{250} or S^{251}) is bearing O-GlcNAc while the other residue is probably important for the binding of the O-GlcNAc transferase to this RING1B region.

We later investigated RING1B O-GlcNAcylation changes during differentiation which may be important for hESC differentiation. hESC were spontaneously differentiated (as previously reported (Maury et al., 2013)) and studied using a multiple reaction monitoring MS (MRM-MS) method we developed to quantify O-GlcNAc peptides (Maury et al., 2014). It is worth noting that such a method is, like all the MS methods, unable to differentiate the O-GlcNAc from its isomer O-GalNAc. Therefore, here, we will use the term of N-acetylated hexosamine (HexNAc) to describe both isomers even though O-GalNAc is mostly found on membrane and extracellular proteins (Brockhausen et al., 2009) and has a low chance of being found on nucleocytoplasmic proteins, such as RING1B. The MRM-MS strategy allowed us to detect the native [($T^{250}S^{251}$)-HexNAc]GNATVDHLSK RING1B peptide

in hESC (Fig. 3A) and quantify the increase of this HexNAcylated peptide following cell treatment with PUGNAc (Figs. S1A–C). Unfortunately, MRM-MS method was unable to detect the other GE[S^{278}-HexNAc]NQMNLDTASEK peptide, probably due to the low stoichiometry of this specific HexNAc peptide. Interestingly, the ratio of the total amount of



RING1B that is HexNAcylated decreased during hESC differentiation, as shown by monitoring the amount of native (TSGNATVDHLSK + HexNAc) peptide during this process (Figs. 3B, C and S1D, E).

These data suggest that O-GlcNAcylation on TSGNATVDHLSK might be functionally important during hESC differentiation. Considering that during differentiation RING1B targets and represses specific developmental-genes, we hypothesized that O-GlcNAcylation of RING1B might regulate RING1B DNA-binding.

O-GlcNAc regulate RING1B DNA-binding

To determine if O-GlcNAcylation regulates RING1B DNA-binding, we carried out a chromatin immunoprecipitation sequencing (ChIP-Seq) experiment (Fig. 4A). To collect sufficient chromatin material for running this experiment from hESC, we expressed recombinantly HA-RING1B and performed a first ChIP to enrich RING1B-bound chromatin followed by a re-ChIP to enrich for O-GlcNAcylated RING1B-bound chromatin. Of note, this strategy might also enrich for other GlcNAcylated proteins being associated with RING1B (such as OCT4, Pol II, Histones and others). Using this strategy, we confirmed the quality of our sequenced DNA (Fig. S2A, B), and identified a total of 6507 genes bound by RING1B among which 1596 genes are bound by O-GlcNAcylated RING1B (Fig. 4B).

Moreover, we found that RING1B bound slightly downstream relative to the transcription start site of genes (Fig. 4C) which is highly similar to a previous report done on mESC Ring1b (Morey et al., 2013). By gene ontology, we found that non-O-GlcNAcylated RING1B-bound genes were mainly related to metabolism and cell cycle processes; whereas O-GlcNAcylated RING1B-bound genes were mainly related to neural differentiation processes (Fig. 4D). Indeed, many neural developmental genes (PAX6, LHX2, RAC2 and others) were bound by O-GlcNAcylated RING1B (Fig. S2C). Interestingly, by comparing our data with reports of ChIP-Seq done specifically on OCT4, SOX2, and NANOG in hESC (Watanabe et al., 2014, p. 63; Gertz et al., 2013); we found that O-GlcNAcylated RING1B preferentially bound to genes co-occupied by the pluripotency core network (Figs. 4E, S2D). These data suggest that O-GlcNAcylated RING1B preferentially binds near stem cell-related genes and more specifically neural developmental-genes.

By taking into account the decrease of RING1B O-GlcNAcylation during differentiation (Fig. 3C); we can finally hypothesize a functional model for the O-GlcNAcylation of RING1B (outlined in Fig. 4F). In this model, hESC maintenance and growth might be promoted by high level of O-GlcNAcylated RING1B which indirectly increases the expression of genes related to cell growth and metabolism. A contrario, neuronal differentiation might be promoted by low level of O-GlcNAcylated RING1B which indirectly increases the expression of genes related to neuronal differentiation.

Discussion

We previously reported that O-GlcNAcylation is important in hESC commitment towards adipose and ectoderm lineages (Maury et al., 2013). However, O-GlcNAc proteins regulating hESC commitment have yet to be characterized. Here, we aimed at extending our understanding of O-GlcNAc regulation occurring during human stem cell differentiation.

Therefore, we revealed for the first time that RING1B, the catalytic core of PRC1, is O-GlcNAcylated in hESC. We revealed that RING1B has at least 2 O-GlcNAcylation sites which are occupied and linked to specific residues: T^{250}/S^{251} and S^{278} respectively. Interestingly, the relative percentage of RING1B O-GlcNAcylation on T^{250}/S^{251} residue decreases during differentiation. We then studied the function of RING1B O-GlcNAcylation using an anti-RING1B ChIP strategy followed by a WGA re-ChIP experiment. Functionally, O-GlcNAcylation on RING1B seemed to modulate RING1B DNA-binding. Non-O-GlcNAc RING1B preferentially bound near genes related to metabolic and cell cycle processes. In contrast, O-GlcNAcylated RING1B preferentially bound near genes related to neural differentiation. Several groups have previously shown that PRC1 can switch gene targets leading to specific cell phenotypes (Rajasekhar and Begemann, 2007; Chen et al., 2011). Our data suggest that O-GlcNAc functions might regulate RING1B DNA-binding and potentially RING1B gene targeting. Therefore, change in RING1B O-GlcNAcylation might be one of the mechanisms enabling PRC1 to switch its gene targets. As RING1B O-GlcNAcylation decreases during hESC differentiation, O-GlcNAcylated RING1B-bound genes (related to neuron differentiation) might be de-repressed in favor of non-O-GlcNAcylated RING1B-bound genes (related to metabolism and cell cycle) (Fig. 3F). This hypothetical mechanism might be necessary during differentiation to 1) slow down cell growth, and 2) direct stem cell differentiation towards neuronal lineage. This is in contrast with maintained hESC where the higher level of O-GlcNAcylated RING1B might prevent neuronal differentiation whereas lower level of non-O-GlcNAcylated RING1B might promote the expression of genes related to metabolism and cell cycle. Such mechanism may be necessary in maintained hESC to promote cell growth.

We previously showed that O-GlcNAc excess in differentiating hESC leads to an 80% mRNA and protein expression decrease for PAX6 (neuronal lineage marker) (Maury et al., 2013). Interestingly, we demonstrated here that PAX6 is bound by O-GlcNAcylated RING1B. This suggests that O-GlcNAcylation increase on RING1B might be one of the

Figure 4 RING1B O-GlcNAcylation regulates RING1B gene targeting. (A) Overview of the re-ChIP process to enrich DNA bound by O-GlcNAcylated RING1B. (B) Veen diagrams of the overlap between genes bound by RING1B and genes bound by O-GlcNAcylated RING1B. (C) Normalized signal of the binding profiles of RING1B (black line) and O-GlcNAcylated RING1B (red line) relative to the transcription start site (TSS) of genes. (D) GO comparison between genes bound by O-GlcNAcylated RING1B and genes bound by non-O-GlcNAcylated RING1B. (E) Co-occupancy between O-GlcNAcylated RING1B, non-O-GlcNAcylated RING1B and OCT4, NANOG, and SOX2 on genes. Data related to the identity of genes bound by OCT4, NANOG or SOX2 were taken from (Watanabe et al., 2014; Gertz et al., 2013). (F) Hypothetical model for the function of O-GlcNAc RING1B on the regulation of hESC differentiation.

reasons for the previously observed effect of O-GlcNAc excess on hESC differentiation.

Mechanistically, O-GlcNAc is probably not directly regulating RING1B binding to chromatin as RING1B is not known to bind to either histone or DNA (Vidal, 2009). Instead, O-GlcNAc might be regulating RING1B binding to PRC1 recruitment subunit (CBX2, 4, 6, 7, 8 and RYBP) which are known to bind to specific gene subsets (Vincenz and Kerppola, 2008; Morey et al., 2013). As RING1B can only bind to one CBXs/RYBP protein (Bezsonova et al., 2009; Wang et al., 2010); each CBXs/RYBP protein will form different PRC1 variants which are thought to target and repress specific gene subsets (Vincenz and Kerppola, 2008; Morey et al., 2013). In addition, RING1B O-GlcNAcylation sites appear in a region (from I^{248} to P^{324}) previously reported as mediating the exclusive binding of RING1B to CBX7 and RYBP (Bezsonova et al., 2009; Wang et al., 2010). Altogether, these data suggest that RING1B O-GlcNAcylation might regulate RING1B affinity for PRC1 recruitment subunit (CBXs/RYBP) ultimately modulating PRC1 DNA-binding.

In summary, we report for the first time that a major epigenetic tool, RING1B, is modified by O-GlcNAc. Our data suggest that RING1B O-GlcNAcylation may regulate PRC1 DNA-binding and might be an essential regulator of stem cell growth and differentiation.

Author contributions

JJPM designed, performed, analyzed the experiments, and wrote the paper. DN and XB performed and analyzed the mass-spectrometry-related experiments. CAEF analyzed the ChIP-related experiments. MB and ABC designed the experiments, analyzed the data and wrote the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.06.007.

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