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The *in silico* Interplay Between Post-Translational Modification in Histone H4 and Its Role in Mouse Spermatogenesis

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Abstract.- The nucleosome is the fundamental repeating unit of chromatin, which consist of histone proteins (H2A, H2B, H3 and H4) wrapped by 147 base pairs of DNA. Post-translational modifications (PTMs) of histones (including acetylation, phosphorylation and methylation) regulate chromatin structure and function. The combination of PTMs of histone proteins regulates various cellular pathways. Phosphorylated H2A and H4 are found in mouse spermatogenesis, and correlates with both mitotic/meiotic chromosome condensation and displacement of histone proteins from the nucleosome structure. Another equally abundant PTM is *O*-GlcNAc modification of Ser and/or Thr in cytoplasmic and nuclear proteins. Some Ser and/or Thr residues, are also available to phosphorylation (Yin Yang sites) in addition to *O*-GlcNAc modification, which occurs in an inverse manner. By using computational methods *in silico* Yin Yang sites in H4 are proposed, and the interplay between phosphorylation, *O*-GlcNAc modification, acetylation and phosphorylation is investigated. The *in silico* results suggest that *O*-GlcNAc modification play a role in spermatogenesis in mammals.

Keywords: Phosphorylation, *O*-GlcNAc modification, acetylation, methylation, spermatogenesis.

INTRODUCTION

Regulation of protein function is critically controlled by posttranslational modifications (PTMs) such as phosphorylation, glycosylation and acetylation. These modifications are temporary and can be difficult to detect experimentally. Bio-informatic tools are very useful for determination of PTMs in proteins. These methods are highly accurate, and can help in exploring structural and functional changes, and ultimately suggest experiments for the verification of functional variants.

Core histone proteins (H2A, H2B, H3 and H4) are evolutionarily and conserved proteins, which together with the DNA form the nucleosome structure and are the basic building blocks of the chromatin structure. Their *N*-terminal tails protrude the compact chromatin structure where they become accessible to different modifications such as acetylation, methylation and phosphorylation. During progression of the cell cycle different modifications of histone proteins play a crucial role. From late G2 phase through telophase the linker histone H1 and core histone H3 are phosphorylated, and are implicated in chromatin condensation and sister chromatid segregation (Hendzel *et al*., 1997). Especially phosphorylation of H3 on Ser 10 (pSer10), 28 and Thr 3, 11 have been found to be important regulators of mitosis (Polioudakia *et al.*, 2004; Preuss *et al*., 2003; Goto *et al*., 1999, 2002). Different modifications of histone H4 have also been shown to play an important role in the progression of the cell cycle. During mitosis and the S-phase of the cell cycle H4 (and H2A) are both phosphorylated on Ser 1 (pSer1) in worms, fly and mammalian cells (Barber *et al*., 2004). Specifically pSer1 of H4 is involved in sporulation in yeast and is present during spermatogenesis in *Drosophila* and mouse (Krishnamoorthy *et al.*, 2006; Wendt and Shilatifard, 2006). Additionally H4 pSer1 has been proposed as a mark for gamete-associated packaging (Krishnamoorthy *et al.*, 2006). Besides being a target of kinases H4 has also been found to become acetylated and methylated. Acetylation occurs on Lys 5, 8, 12 and 16 (acLys5, 8, 12 and 16) (Zhang *et al*., 2002), and methylation at Lys 20 (mLys20) prevents its own acetylation, and play a role in gene activation and repression, chromatin condensation,

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S-phase progression and mitosis (Yang and Mizzen, 2009). The combination or interplay between different modifications in H4 has also been reported to be associated with transcriptional activation. As an example methylated H4 on Arg 3 (mArg3) facilitate subsequent acetylation leading to transcriptional activation, but prior acetylation of H4 prevents methylation and subsequent transcription (Pesavento *et al*., 2008; Wang *et al*., 2001).

Another equally important and abundant modification is *O*-GlcNAc modification that occurs on both cytosolic and nuclear proteins (Comer and Hart, 1999). The addition of an *O*-GlcNAc moiety to the protein backbone is catalyzed by *O*-GlcNAc transferase (OGT) (Slawson *et al*., 2006). *O*-GlcNAc modification and phosphorylation can be reciprocal and may occur on the same or adjacent Ser/Thr residues (Comer and Hart, 1999). These sites are termed Yin Yang sites, as on these sites both kinases and OGT have equal opportunity to add a phosphate or *O*-GlcNAc moiety, respectively. Previously it has been suggested that histone proteins have potential of being *O*-GlcNAc modified, which may control gene expression of immediate early genes (Kaleem *et al*., 2008), prevent apoptosis and DNA fragmentation (Kaleem *et al*., 2006b) and may control specific checkpoints during mitosis (Kaleem *et al*., 2006a).

In the present investigation, we have predicted the possible interplay between phosphorylation, acetylation, methylation and *O*-GlcNAc modification of the mammalian histone H4. Our results suggest that *in silico* interplay between the different PTMs in H4 control condensation of the chromatin structure during spermatogenesis in mammals.

MATERIALS AND METHODS

The sequence data used for the prediction of phosphorylation and *O*-glycosylation potential of histone H4 in *Mus musculus* was retrieved from the SWISS-PROT database (Boeckmann *et al.*, 2003) with primary accession no. P62806 (H4_MOUSE). BLAST search was performed using NCBI database of non-redundant sequences using all default parameters (Altschul *et al.*, 1997). The search results were divided into *vertebrates* and *invertebrates* (Table I). The chosen sequences were multiply aligned using ClustalW2 with all default parameters (Thompson *et al.*, 1994).

Table I.- Different H4 sequences selected for multiple alignment in CLUSTALW2.

Vertebrate		Invertebrate	
Homo sapiens	P62805	*Drosophila melanogaster*	NP_001027382.1
Danio rerio	CAM12247.1	*Oikopleura dioica*	Q8I0Y4.3
Gallus gallus	P62801	*Aedes aegypti*	XP_001657284.1
Bos taurus	NP_776305.1	*Mytilus chilensis*	AAP94670.1
Rattus norvegicus	AAA60735.1	*Culex quinquefasciatus*	EDS41770.1

The potential for phosphorylation and *O*-GlcNAc modification in *Mus musculus* histone H4 was predicted by Netphos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) (Blom *et al.*, 1999), DIPHOS 1.3 (http://www.ist.temple.edu/DISPHOS) (Iakoucheva *et al.*, 2004) and YinOYang 1.2 (http://www.cbs.dtu.dk/services/YinOYang/) (Gupta and Brunak, 2002), respectively. The potential for acetylation and methylation was predicted by using LysAcet 1.1 (Li *et al*., 2009), and Memo (a computational method for prediction of protein methylation modifications in proteins) (Chen *et al*., 2006).

These bio-informatic tools are neural networks, which are composed of a large number of highly interconnected processing elements (simulated neurones) that work in parallel to solve a complex problem. These networks are trained by sequence patterns of modified and non-modified proteins. Artificial neural networks receive many inputs and give one output. NetPhos 2.0 was developed by training the neural networks with phosphorylation data from Phosphobase 2.0. A threshold value of 0.5 is used by Netphos 2.0 to determine possible potential for phosphorylation. In case of YinOYang 1.2 employs the sequence data to train a jury of neural networks on 40 experimentally determined *O*-GlcNAc acceptor sites for recognizing the sequence context and surface accessibility. Furthermore this method has the capability to predict the sites known as Yin Yang sites that can be *O*-GlcNAc modified and alternatively phosphorylated. The threshold value

used by YinOYang 1.2 varies depending upon surface accessibility of the different amino acid residues. False negative sites are identified as well, by coupling conservation status and modification potential of the two methods.

RESULTS AND DISCUSSION

Different PTMs acts as modulators of proteins, and are expected to function in the same manner as in their ortologs from phylogenetically-related organisms. Thus PTMs are often inferred by the similarity with a model organism. This can only be true if the protein is present in both organisms, and the sequence in the vicinity of the PTM is conserved in both organisms. The histone proteins are highly conserved and are of evolutionary importance.

In the current study the potential for phosphorylation, *O*-GlcNAc modification, acetylation and methylation and the interplay between these modifications are investigated utilizing different bio-informatic tools. In Table II are the results for the prediction of phosphorylation, *O*-GlcNAc modification, Yin Yang, acetylation and methylation sites tabulated and illustrated in Figs. 1a-c. The potential phosphorylation, *O*-GlcNAc modification, Yin Yang sites are conserved in vertebrates and invertebrates (Fig. 2). In H4 3 phosphorylation sites were positively predicted by the Netphos 2.0 and DIPHOS 1.3 server and in case of *O*-GlcNAc modification only 1 site was predicted as a positive modification site by the YinOYang 1.2 server (Table II). Furthermore Yin Yang sites were also predicted, but none were positively predicted by the YinOYang 1.2 server. In case of Ser 1, it is an experimentally known phosphorylation site, and it was predicted as a positive phosphorylation site by DIPHOS 1.3. Furthermore it showed a potential for *O*-GlcNAc modification very close to the threshold, suggesting that it is a false negative Yin Yang. A false negative site is a conserved residue that shows a potential for phosphorylation and a potential for *O*-GlcNAc modification very close to the threshold value or vice versa. On these sites OGT and kinases may have an equal accessibility to modify the specific site. The first six amino acids (MSGRGK) of histone H4 and H2A are identical in *Mus musculus* and are also highly conserved throughout evolution. When the potential for *O*-GlcNAc modification in *Mus musculus* H2A was predicted by YinOYang 1.2 server, it was found that Ser1 was positive predicted *O*-GlcNAc modification site. These results suggest that Ser1 in H4 also can be a potential *O*-GlcNAc modification site, hence a Yin Yang site.

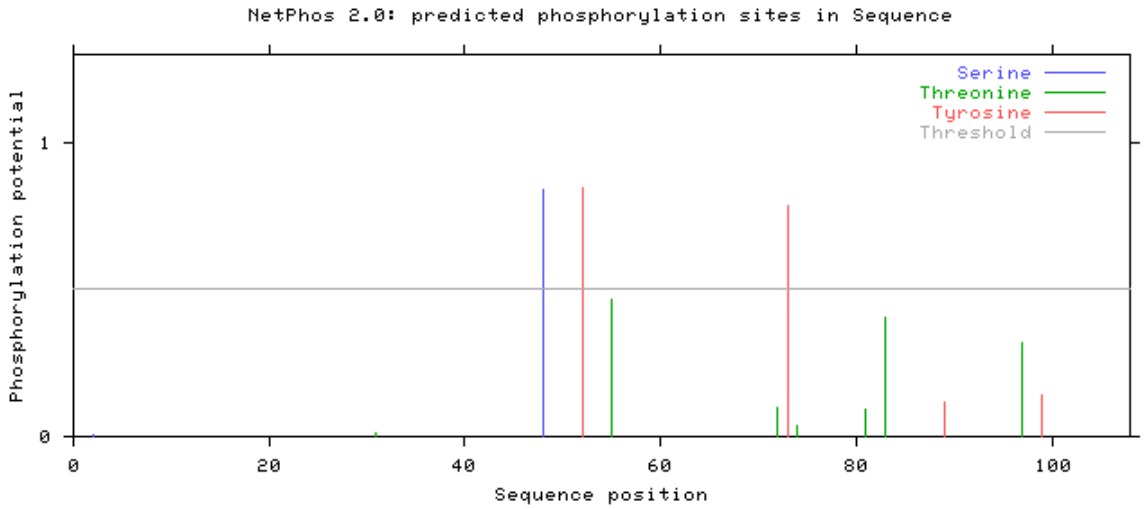
Table II.- Result for the potential of phosphorylation, *O*-GlcNAc modification, Yin Yang, acetylation and methylation *in silico* in *Mus musculus* histone protein H4. (Histone H4 has an initiator methione at position 1, which has been removed)

Positive predicted phosphorylation sites (Netphos)	Ser 47, Tyr 51,72
Positive predicted phosphorylation sites (DIPHOS)	Ser 1, Tyr 72
Positive predicted *O*-GlcNAc modification site	Thr 71
False negative Yin Yang site	Ser 1
Positive predicted acetylation sites	Lys 12,16,20,43,77,79,91
Positive predicted methylation sites	Arg 3,40,45,55 Lys 5,8,20,59,79

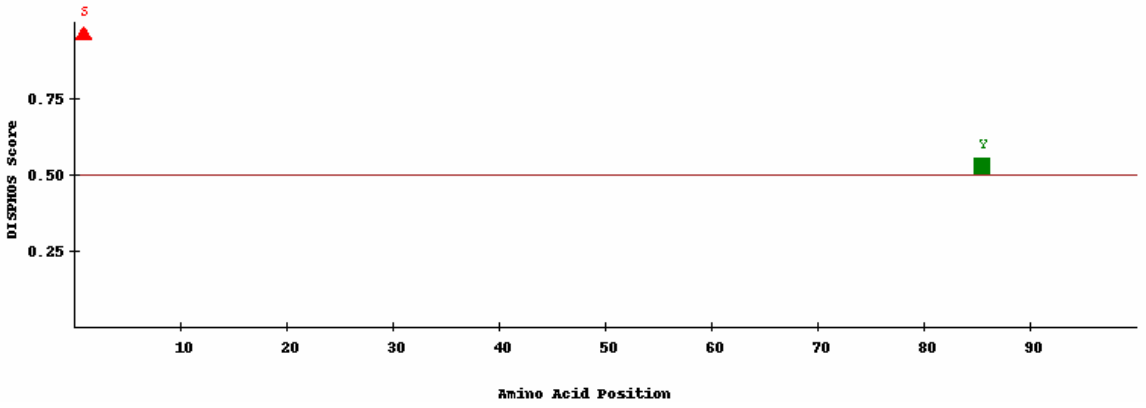
Phosphorylation of H4 on Ser1 is cell cycle dependent. During S-phase newly synthezised H4 becomes phosphorylated on Ser1, and during mitosis both H4 and H2A are phosphorylated on their respective Ser1 residue (Barber *et al.*, 2004). Furthermore, both H2A and H4 share the same sequence on their first 6 residues, it suggests their functional similarity in mammalian cells.

The role of different covalent modifications of histone proteins during the progression of the cell cycle is well known. In case of histone H3 phosphorylation on Ser10, it has been detected in late G2 phase. In metaphase its phosphorylation is highest and persists until the completion of telophase (Goto *et al.*,1999; Hendzel *et al.*, 1997). In case of H4 pSer1 (and H2A pSer1) phosphorylation was detected during early prophase, anaphase and declined during telophase in the same manner as H3 pSer10 (Barber *et al.*, 2004). These findings suggest that phosphorylated H2A, H3 and H4 play a definite role in condensation and segregation of mitotic chromosomes. Furthermore

(a)



(b)



(c)

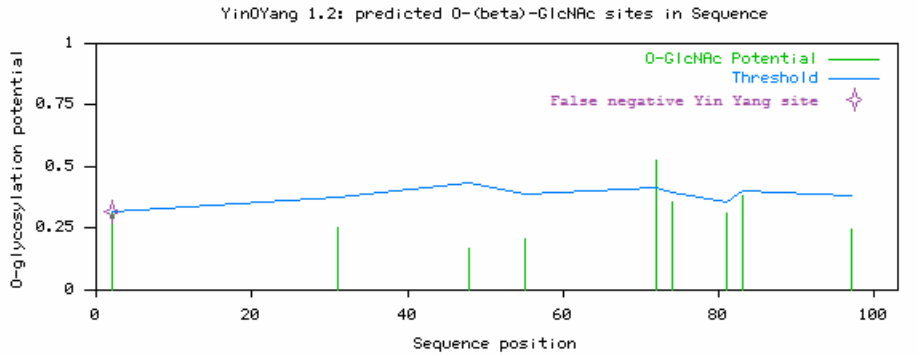


Fig. 1. (a) Graphical representation of the prediction by Netphos 2.0 of phosphorylation in *Mus musculus* H4 on Ser, Thr and Tyr residues. (b) Graphical representation of the prediction by DIPHOS 1.3 of phosphorylation in *Mus musculus* H4 on Ser, Thr and Tyr residues. (c) The potential of *O*-GlcNAc modification and prediction of Yin Yang sites in *Mus musculus* H4. The light blue horizontal wavy line shows threshold for modification potential, the green vertical lines show the potential of Ser, Thr residues for *O*-GlcNAc modification and the asterisk indicates the false-negative Yin Yang site.

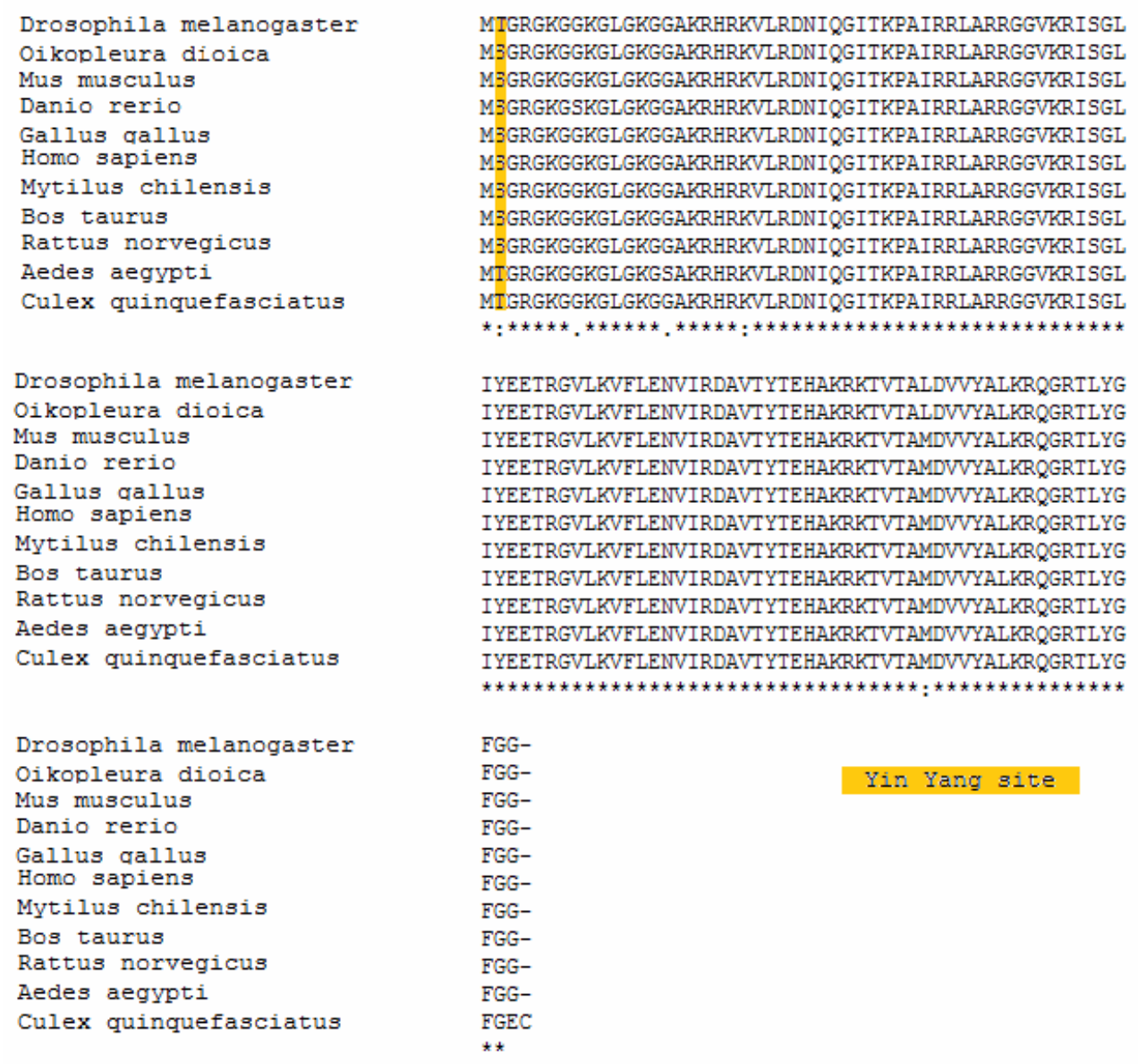


Fig. 2. Multiple alignments of six vertebrate and five invertebrate sequences. The *consensus* sequence is highlighted by asterisk, conserved substitution by a double dot and semiconserved substitution by a single dot.

phosphorylated H2A and H4 also participate in the deposit of newly synthesized histone, as H2A and H4 are hyperphosphorylated during S-phase. H4 pSer1 and H2A pSer129 also play a role in repair of damaged DNA double-strand breaks in yeast (Cheung *et al.*, 2005). Various combinations of histone modifications may serve as signals, and specific combination of PTMs may determine the final outcome. As in the case of H3 the combination of phosphorylation, *O*-GlcNAc modification, acetylation and methylation *in silico* controls the expression of immediate early genes (Kaleem *et al.*, 2008). Furthermore a reduced level of H4 acLys12 leads to decreased H3 pSer10 (Ciurciu *et al.*, 2008), and H4 acLys12 is co-localized with acetylated and mLys4 H3 (Valls *et al.*, 2005). H4 mArg3 subsequently leads to acLys8 and acLys12, which are important regulators of transcription (Wang *et*

al., 2001). *O*-GlcNAc modification is also known to control transcription and translation (Yang *et al.*, 2002: Comer and Hart, 2000). The interplay between phosphorylation and *O*-GlcNAc *in silico* in histone proteins H2B and H3 has been investigated, which showed that interplay regulates mitosis, apoptosis and transcription in mammals (Kaleem *et al.*, 2008; Kaleem *et al.*, 2006a,b).

In this work Ser1 in H4 is *in silico* a false-negative Yin Yang site, which means that both kinases and OGT are capable of modifying this residue and inversely regulate the chromatin structure. During spermatogenesis in vertebrates histone proteins (specifically H4) are hyperacetylated, and become displaced by protamines (Govin *et al.*, 2004; Lahn *et al.*, 2002). These results suggest that both phosphorylation and acetylation co-exist on H4 during spermatogenesis in mammals. Furthermore phosphorylated Ser/Thr next to Lys prevents its methylation like in the case of H3 Lys9 Ser10 (Govin *et al.*, 2004), which shows that acetylation and methylation can be inverse to each other. During spermatogenesis H3 is phosphorylated on Ser10 and methylated on Lys4 (Slany, 2009; Godmann *et al.*, 2007). A methyltransferase MLL5 methylates Lys4 in H3. MLL5 (or in general MLL transferases) are incorporated into macrocomplex with histone methyltransferase and acetyltransferase function (Slany, 2009). These complexes are found at promoters of transcriptionally active genes. When MLL is combined with the acetyltransferase p300/CBP complex, they work together to regulate transcription. MLL is known to methylate H3 on MLL5, which is activated by *O*-GlcNAc modification in its SET domain (Fujiki *et al.*, 2009). The combinatorial regulation of histone proteins shows that pSer1 and acetylated H4 co-exist during spermatogenesis in mammals, and has similar timing and localization as H3 pSer10 and mLys4. But pSer1 in H4 persist late into sperm differentiation and gametogenesis, where pSer10 in H3 disappears. pSer1 H4 is reduced with replacement of histone proteins by protamines. Furthermore H4 acetylated also persist during this process and also plays a role in displacement of histone proteins (Meistrich *et al.*, 2005). When H4 Ser1 and H3 Ser10 is *O*-GlcNAc modified *in silico* spermatogenesis will not proceed, because pSer1 H4 is an evolutionary mark for this process. Furthermore as MLL5 is activated by *O*-GlcNAc modification, it suggest that OGT play role a dual role in spermatogenesis, as it activates MLL5 to methylate H3 Lys4 and prevents spermatogenesis by *O*-GlcNAc modifying H3 and H4. In conclusion this *in silico* study suggests that the interplay between *O*-GlcNAc modification, phosphorylation, acetylation and methylation of H3 and H4 control spermatogenesis in mammals. And in future it will be important to determine the impact of *O*-GlcNAc modification on the interplay between these modifications, as this modification might play a role in fertilization.

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