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Functional Regulation of DNA Binding of FOXO1 by Post Translational Modifications: *In silico* Study

Asma Butt,[1] Afshan Kaleem, [1,2] Zeeshan Iqbal, [1] Evelyne Walker-Nasir, [1] Muhammad Saleem, [3] Abdul Rauf Shakoori[4] and Nasir-ud-Din[1,5]

[1]*Institute of Molecular Sciences & Bioinformatics, Lahore, Pakistan*
[2]*Lahore College for Women University, Lahore, Pakistan*
[3]*Department of Botany, University of the Punjab, Lahore, Pakistan*
[4]*School of Biological Sciences, University of the Punjab, Lahore, Pakistan*
[5]*HEJ Research Institute of Chemistry, University of Karachi, Karachi, Pakistan*

Abstract.- The transcription factor Forkhead box O 1 (FOXO1) is a key regulator of metabolic processes such as regulation of the cell cycle and cancer. Different post translational modifications (PTMs) such as phosphorylation, glycosylation, acetylation, methylation and ubiquitination control the structure, function regulation and subcellular localization of FOXO1. In this work the role of different modifications and their interplay, involving the regulating the transcriptional activity of FOXO1, is investigated by using various bio-informatic tools. Amongst these is the YinOYang prediction method, which predicts Yin Yang sites in proteins (sites, where *O*-glycosylation and phosphorylation may compete with each other). Moreover acetylation and methylation may also work together to regulate FOXO1 transcriptional activity. This study suggest that phosphorylation and acetylation deactivate FOXO1's transcriptional activity by disrupting binding between DNA and FOXO1, and promote ~~its~~ cytoplasmic localization and degradation of the FOXO1 transcription factor. Furthermore, glycosylation and methylation increase the DNA binding affinity and enhance nuclear accumulation of FOXO1 and promote transcriptional activity. Thus this *in silico* work suggests that different modifications play an important role in the regulation of FOXO1's transcriptional activity and its target genes.

Key words: FOXO1 transcription factor, Yin Yang, *O*-GlcNAc, phosphorylation, post translational modification.

INTRODUCTION

FOXO is a subfamily of Forkhead Box (FOX) transcription factors, and is further divided into FOXO1, FOXO3, FOXO4 and FOXO6 (Maiese *et al.,* 2008). The FOXO transcriptional factors play a critical role in many biological processes such as regulation of cell cycle, oxidative stress, DNA repair, longevity and cancer (Ouyang *et al.,* 2009; Yuan *et al.,* 2008; Kuo *et al.,* 2008; Hoekstra *et al.,* 2008; Lehtinen *et al.,* 2006).

FOXO1 also known as forkhead in rhabdomyosarcomas (FHKR) and is a downstream target of insulin signaling pathway. FOXO1 is an important regulator of cellular processes such as apoptosis, aging and stress response (Cheng and White, 2010; Kuo *et al.,* 2008; Berry *et al.,* 2009; Bartek and Lukas 2006; D'Alessandris *et al.,* 2004; Kim *et al.,* 2008). It is ubiquitously expressed, but is highly expressed in pancreatic beta cells, adipose tissues and muscles. FOXO1 contains a conserved forkhead DNA binding domain (DBD) also known as winged helix, which comprises 3 beta sheets, 3 alpha helixes and 2 loops. The FOXO1 transcription factor binds to the consensus DNA sequence TTGTTTTG in the promoter through its DBD (Cheng and White, 2010).

FOXO1 is regulated through interplay between different post translational modifications (PTMs) like phosphorylation, acetylation, methylation, glycosylation, and ubiqutination occurring in or near the DBD (Brent *et al.,* 2008). These modifications affect the transcriptional activity, DNA binding affinity and localization of FOXO1 transcriptional factor in nucleoplasm or cytoplasm (Van Der Heide *et al.,* 2004). The Ser/Thr kinase Akt induces phosphorylation of FOXO1, and inhibits the binding between DNA and FOXO1, which increases cytoplasmic localization of FOXO1 from the nucleus. In the cytoplasm

* Corresponding author: professor_nasir@yahoo.com, prof_nasir@yahoo.com, Web: www.imsb.edu.pk.
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phosphorylated FOXO1 is ubiquitinated, which leads to its degradation (Huang *et al.,* 2006; Matsuzaki et al., 2003).

FOXO1 is activated through phosphorylation in pre-prandial, oxidative stress and insulin resistance conditions, while in postprandial and insulin sensitive conditions its activity is inhibited by dephosphorylation (Dong *et al.*, 2010). Phosphorylated and dephosphorylated FOXO1 both causes disruption in metabolism homeostasis. The hyperphosphorylated FOXO1 transcription factor during insulin resistance may cause hyperglycemia and dyslipidemia (Cheng and White, 2010), whereas hypo-activated FOXO1 may disrupt T cell homeostasis (Ouyang *et al.,* 2009) and B cell proliferation (Kitamura *et al.,* 2005).

Glycosylation plays an important role in cell signaling, transcriptional and translational activity, cell survival, and cellular immunity. Glycosylation is a dynamic modification like phosphorylation, which takes place on Ser and/or Thr residues in the protein. A dynamic interplay between these two modifications, also known as Yin Yang sites, have been shown to control biological processes such as transcription, apoptosis etc. Furthermore *O*-GlcNAcylation of FOXO1 increases its transcriptional activity (Kuo *et al.,* 2008).

In this study the interplay of different PTMs especially glycosylation and phosphorylation, acetylation and methylation, phosphorylation and methylation inversely regulates the activity and translocation of FOXO1 are studied using different bio-informatics tools. The *in silic*o investigation suggests that the interplay between phosphorylation and glycosylation, acetylation and methylation on specific sites may control the transcriptional activity of FOXO1 and hence regulate various metabolic processes like beta cell proliferation, energy homeostasis, glucose consumption in blood, inflammation, oxidative stress responses.

MATERIALS AND METHODS

The sequence of FOXO1 of *Mus musculus* was retrieved from Swiss-Prot with an accession number Q9R1E0, Swiss-Prot ID FOXO1_MOUSE and gene name FOXO1 (Boeckmann *et al.,* 2003). Blast search was performed using NCBI data to find the orthologues (Altschul *et al.,* 1997). The search resulted in 12 selected orthologues (Table I) each having E–value zero to 3e-151 and similarity of 52-100%. The selected orthologues were multiply aligned using ClustalW2 with default parameters.

Table I.- The accession # of 12 orthologues of FOXO1

Accession #	Specie
Q9R1E0.1	*Mus musculus*
NP_001178775.1	*Rattus norvegicus*
Q810W5.1	*Spermophilus tridecemlineatus*
XP_583090.4	*Bos taurus*
AAM19156.1	*Sus scrofa*
NP_002006.2	*Homo sapiens*
XP_522749.2	*Pan troglodytes*
NP_989659.1	*Gallus gallus*
NP_001008016.1	*Xenopus (Silurana) tropicalis*
NP_001086417.1	*Xenopus laevis*
NP_001070725.2	*Danio rerio*
NP_001153936.1	*Oryzias latipes*

The glycosylation and Yin Yang sites of FOXO1 in *Mus musculus* were predicted using YingOYang 1.2 (http://www.cbs.dtu.dk/services/YinOYang/). The phosphorylation sites were determined using NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos 2.0/) and DIPHOS 1.3 (http://www.ist.temple.edu/disphos/). The potential acetylation and methylation sites were predicted using PAIL (http://bdmpail.biocuckoo.org/prediction.php) and MeMo (http://www.bioinfo.tsinghua.edu.cn/~tigerchen/memo.html), respectively.

NetPhos 2.0 (Blom *et al.,* 1999) and DIPHOS 1.3 (Iakoucheva *et al.,* 2004) both are neural networks and trained by dataset of patterns of both modified and non modified proteins. NetPhos 2.0 uses phosphorylation data from phosphobase while DIPHOS 1.3 uses sequence and disordered information phosphorylated proteins obtained from Phospho.ELM database. PAIL (Li *et al.,* 2006) predict the acetylation on internal Lys using Bayesian Discriminant Method while MeMo is working on support vector machine method and predicts methylation on Lys and Arg residues. YinOYang 1.2 is a neural network and trained a dataset of 40 experimentally known glycosylation sites to recognize the sequence context and surface

accessibility. YinOYang can also predict the Yin Yang sites with a variable threshold.

RESULTS AND DISCUSSION

The interplay between different PTMs such as phosphorylation and glycosylation, phosphorylation and acetylation, phosphorylation and methylation is known to play a key role in the functional regulation of different proteins (Kaleem *et al.*, 2009, 2010). In this work the internal relationship of above mentioned modifications in FOXO1 has been investigated using various bio-informatics tools.

The activity of FOXO1 is controlled by different PTMs, and hence regulates various metabolic processes. In this study the role of PTMs in FOXO1 of *Mus musculus* has been investigated and compared with their evolutionary status in different orthologues. The multiple alignment of FOXO1 (Fig. 1) shows that all orthologues have very high similarity and are almost conserved in their DNA binding region (156-232 amino acids). The DIPHOS 1.3 server predicted 19 phosphorylation sites (13 sites are conserved (C) and 6 sites are non-conserved (NC)), while the Netphos 2.0 server predicted 60 potential phosphorylation sites (32 sites are C, 25 are NC, and 3 are conserved substitution (CS)) (Table II). The YinOYang 1.2 server has predicted 44 potential *O*-GlcNAc sites (19C, 23NC and 2CS). Furthermore 20 potential Yin Yang sites were predicted (11 Yin Yang sites are C, 6 are NC and 3 are CS among different orthologues) (Fig. 1). Amongst all the potential predicted phosphorylation sites only seven are experimently determined *in vitro* and *in vivo*, while only four *O*-glycosylation sites are determined experimentally *in vitro* (Hatta *et al.,* 2009; Housley *et al.,* 2008; Yamagata *et al.,* 2008; Rena *et al*., 2002) (Table II). *In vitro* and *in vivo* analysis showed S209 as target site for Mammalian sterile 20-like 1 (MST1) induced phosphorylation, but neither Netphos 2.0 nor DIPHOS 1.3 has predicted S209 as positive potential site. The interplay between glycosylation and phosphorylation regulates the transcriptional activity of FOXO1 transcription factor by increasing or decreasing its DNA binding affinity, as these modifications are inversely regulated. Phosphorylation of FOXO1 occurs in response to insulin, which increases the negative charge on FOXO1, thereby disrupting its DNA binding and increases the nuclear exclusion. Once in the cytoplasm phosphorylation of FOXO1 promotes poly-ubiquitination, which results in degradation. FOXO1 is phosphorylated using different kinases, all kinases except cyclin dependent kinase 1 (CDK1) and MST1 reduces the DNA binding affinity and nuclear localization (Yuan *et al.,* 2008; 2009; Huang *et al.,* 2006). FOXO1 is glycosylated through hexoseamine glycosylated pathway in insulin resistance and oxidative stress conditions (Housley *et al.,* 2008).

In response to insulin signaling FOXO1 undergoes phosphorylation at T24, S316, S253 by protein kinase B also known as Akt, which inhibit FOXO1 transcriptional activity. Furthermore phosphorylation of FOXO1 decreases its interaction with DNA and hence reduces the expression of its targeted genes (Matsuzaki *et al.*, 2005). This chromosomal translocation promotes interaction of FOXO1 with 14-3-3 proteins. S316 is also a potential Yin Yang site predicted by YinOYang server. Although experimental analysis of FOXO1 have shown that S316 is not a potential glycosylation site, but mutating of S316 to alanine lead to an increase of glycosylation at T314 (also a potential Yin Yang site) suggesting an interplay between glycosylation and phosphorylation at distinct sites (Housley *et al.,* 2008). Akt mediated phosphorylation of FOXO1 protein activates nuclear factor kappa B (Nf-kB), which mediates inflammation induced by oxidative stress during aging. Thus by inhibition of FOXO1 phosphorylation during caloric restriction causes an increase in expression of the catalase gene and suppression of oxidative stress induced inflammation (Kim *et al.,* 2008). Akt induced phosphorylation at S316 causes casein kinase 1 (CK1) to phosphorylate S319 and then stimulate phosphorylation at S322 (Rena *et al*., 2002). Dual-specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1A) phosphorylate S326 of FOXO1 in non-insulin stimulated cells and reduces the nuclear localization and transactivation of FOXO1 transcription factor (Woods *et al.,* 2001). Mammalian sterile 20-like 1 (MST1) and CDK1

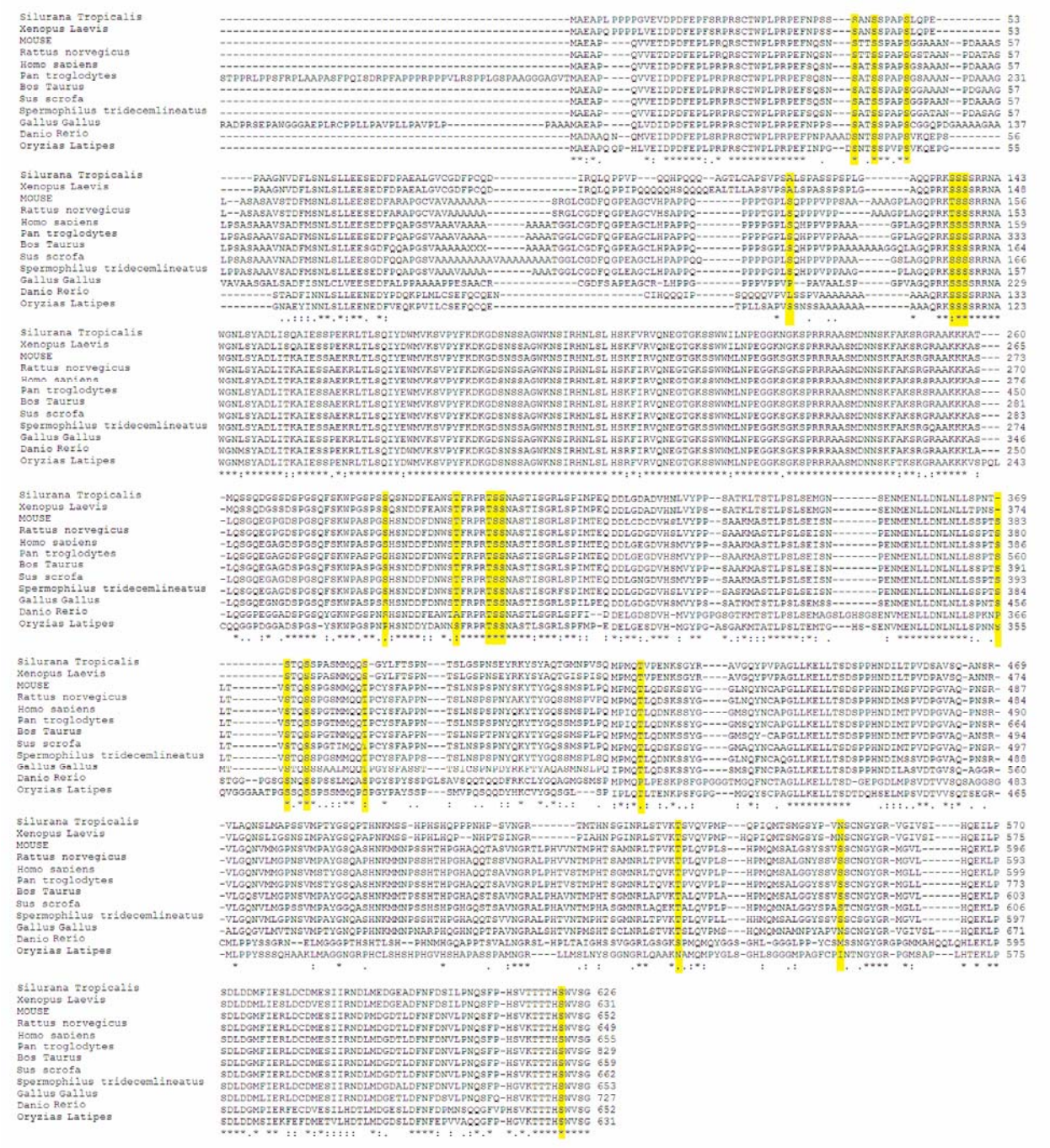

Fig. 1. Multiple sequence alignment of 7 Mammalian and 5 Non-Mammalian species using ClustalW2. Yellow colors indicated the potential Yin Yang sites

induced phosphorylation at S209 and S246 respectively, of FOXO1 and reduce its interaction with 14-3-3 protein and enhance nuclear accumulation in neurons and promote neuron cell death (Yuan *et al.,* 2008, 2009), while the CDK2 induced phosphorylation at S246 is known to decrease nuclear localization and inhibit transcriptional activity (Huang *et al.,* 2006).

Table II.- The potential predicted and experimentally known sites of phosphorylation, glycosylation and Yin Yang sites.

Position	Amino Acid	DIPHOSPHO 2.0	NetPhos 1.2	Yin Yang site	*O*-GlcNAc sites	Experimentally known	Conservation status
22	S	-	+	-	-	-	C
24	T	+	-	-	-	+	C
37	S	-	+	+	+	-	C
38	T	-	-	-	+	-	C
39	T	-	-	-	+	-	NC
40	S	-	+	+	+	-	C
41	S	-	-	-	+	-	C
45	S	+	+	+	+	-	C
57	S	+	-	-	-	-	NC
62	S	-	+	-	-	-	NC
65	S	-	+	-	-	-	NC
66	T	-	-	-	+	-	NC
73	S	-	+	-	-	-	NC
78	S	-	+	-	-	-	NC
122	T	-	-	-	+	-	NC
126	S	+	+	+	+	-	NC
134	S	+	-	-	+	-	NC
149	T	-	+	+	+	-	CS
150	S	+	+	+	+	-	C
151	S	-	+	+	+	-	C
152	S	-	+	-	-	-	C
161	S	-	+	-	-	-	C
172	S	-	+	-	-	-	C
200	S	-	+	-	-	-	C
209	S	-	-	-	-	+	C
228	T	-	+	-	-	-	C
243	S	+	+	-	-	-	NC
246	S	+	+	-	-	+	C
253	S	+	+	-	-	+	C
263	S	+	+	-	-	-	C
273	S	+	-	-	-	-	CS
276	S	+	+	-	-	-	NC
284	S	+	+	-	-	-	C
287	S	+	-	-	-	-	C
295	S	+	+	-	-	-	C
298	S	+	+	+	+	-	NC
300	S	-	+	-	-	-	C
309	T	-	+	+	+	-	CS
314	T	-	+	+	+	+	C
315	S	-	-	+	+	-	C
316	S	+	+	+	+	+	C
319	S	+	+	-	-	+	C
322	S	+	-	-	+	+	C
326	S	-	+	-	-	+	C
330	T	-	+	-	-	-	C
348	S	-	-	-	+	-	C
354	S	-	-	-	+	-	CS
358	S	-	+	-	-	-	C
360	S	-	+	-	-	-	CS
363	S	-	+	-	-	-	NC
380	S	-	+	-	-	-	NC
383	S	-	+	+	+	-	NC

Continued

Position	Amino Acid	DIPHOSPHO 2.0	NetPhos 1.2	Yin Yang site	*O*-GlcNAc sites	Experimentally known	Conservation status
385	**T**	-	-	-	+	-	**NC**
387	**S**	-	+	+	+	-	**C**
388	**T**	-	-	-	+	-	**NC**
390	**S**	-	+	+	+	-	**C**
391	**S**	-	+	-	-	-	**C**
394	**S**	-	+	-	-	-	**NC**
399	**T**	-	+	+	+	-	**CS**
403	**S**	-	-	-	+	-	**NC**
409	**T**	-	-	-	+	-	**CS**
410	**S**	-	-	-	+	-	**NC**
413	**S**	-	+	-	-	-	**NC**
415	**S**	-	+	-	-	-	**NC**
422	**T**	-	-	-	+	-	**NC**
427	**S**	-	+	-	-	-	**NC**
429	**S**	-	+	-	-	-	**NC**
438	**T**	-	+	+	+	-	**NC**
444	**S**	-	+	-	-	-	**NC**
465	**S**	-	+	-	-	-	**C**
467	**S**	-	+	-	-	-	**NC**
475	**S**	-	+	-	-	-	**NC**
486	**S**	-	-	-	+	-	**NC**
528	**T**	-	+	-	-	-	**NC**
546	**T**	-	-	-	+	-	**NC**
553	**T**	-	+	-	-	-	**NC**
557	**T**	-	+	+	+	-	**NC**
564	**S**	-	-	-	+	-	**NC**
570	**S**	-	-	-	+	-	**NC**
576	**S**	-	-	-	+	-	**NC**
577	**S**	-	-	-	-	-	**NC**
579	**S**	-	+	+	+	-	**NC**
597	**S**	-	+	-	-	-	**C**
613	**S**	-	+	-	-	-	**CS**
637	**S**	-	-	-	+	-	**NC**
641	**S**	-	+	-	-	-	**NC**
644	**T**	-	-	-	+	-	**C**
645	**T**	-	-	-	+	+	**C**
646	**T**	-	+	-	-	-	**C**
648	**S**	-	+	+	+	-	**C**
651	**S**	-	-	-	+	+	**C**

Footnote: C, conserved, NC, not conserved; CS, conserved substitution.

During insulin resistant conditions *O*-linked *N*-acetylglucosamine transferase (OGT) glycosylate FOXO1, which inhibits phosphorylation of FOXO1, and thus increases its nuclear localization and enhances the transcriptional activity of FOXO1 (Housley *et al.,* 2009; Kuo *et al.,* 2008). The residues T314, S547, T645, and S651 are experimentally known glycosylation sites (Housley *et al.,* 2008). In insulin resistant conditions, FOXO1 glycosylation increases the activities of the glycogenic proteins in liver, and over expression of glycosylated FOXO1 may lead to gluconeogenesis. In insulin induced conditions FOXO1 is excluded from the nucleus resulting in reduction of gluconeogenesis. Muscles overexpression of FOXO1 may lead to lipogenesis as it increases the lipid contents in the muscles (Cheng and White 2010). In starvation, inhibition of Akt pathway causes increase glycosylation of FOXO1 in liver causes hyperglycemia (Kuo *et al.,* 2008). Thus phosphorylation and glycosylation plays an important role in regulation of transcriptional

activity of FOXO1.

Acetylation and methylation also play an important role in various transcription factors such as FOXO1 (Yamagata *et al.,* 2008; Hatta *et al.,* 2009). Acetylation of FOXO1 promotes phosphorylation and inhibit glycosylation, while methylation at sites R248 and R250 of FOXO1 are known to block Akt mediated phosphorylation of FOXO1, and inhibits nuclear exclusion of FOXO1 (Yamagata *et al.,* 2008) and thus increases the glycosylation. Potential acetylation and methylation sites are predicted using bio-informatic tools. 19 sites (15C and 4NC) for acetylation and 6 sites for methylation have been positively predicted by using PAIL and MeMo respectively (Table III). Only 1 methylation site and 3 acetylation sites are experimentally known (Yamagata *et al.*, 2008).

In oxidative stress, acetylation of FOXO1 occurs at lysines K242, K245 and K262 by p300. Acetylation enhances phosphorylation of FOXO1 at S253, which decreases the DNA binding and increases the rate of phosphorylation at other Akt induced phosphorylation sites (Hatta *et al.,* 2009; Matsuzaki *et al.,* 2005). Thus phosphorylation and acetylation co-exist to regulate the function and localization of transcription factor. In the cytoplasm acetylation inhibits ubiquitination, thus preventing FOXO1 degradation (Kitamura *et al.,* 2005). If phosphorylated FOXO1 is not acetylated, it undergoes SCF (Skp1-Cullin1-F-box protein)-Skp2 (S phase kinase-associated protein 2) mediated ubiquitination and degradation. So acetylation and ubiquitination compete for the survival of FOXO1 transcriptional factor in cytoplasm.

Table III.- Potential predicted sites of acetylation and methylation using PAIL and MeMo.

PTM	Position
Acetylation	K148, K176, K195, K197, K207, K242, K245, K259, K262, K269, K270, K271, K351, K420, K443, K460, K512, K594, K643
Methylation on Arginine	R98, R250, R264, R266
Methylation on Lysine	K207, K270

The importance of interplay of different PTMs in regulation of transcriptional activities of various transcriptional factors has been described by us previously (Kaleem *et al.,* 2008, 2009, 2010; Nasir-ud-Din *et al.,* 2010). Phosphorylation and glycosylation interplay plays an important role in regulation of transcriptional activity of various transcriptional factors. FOXO1 binding affinity to DNA and translocation is also shown to be regulated by this PTM switching. The phosphorylation of FOXO1 at S316 inhibits the glycosylation at T314 and thus decreasing the DNA binding affinity. In the similar fashion, glycosylation at T314 causes an increase in DNA binding and nuclear accumulation while a decrease in Akt induced phosphorylation at S316. This study has shown an overview of the FOXO1 binding regulation with DNA through interplay of PTMs mainly the phosphorylation-glycosylation and acetylation-methylation, and methylation-phosphorylation which compete for the same or neighboring sites (Fig. 2). FOXO1 is required for proper functioning of many cellular biochemical processes such as metabolism and

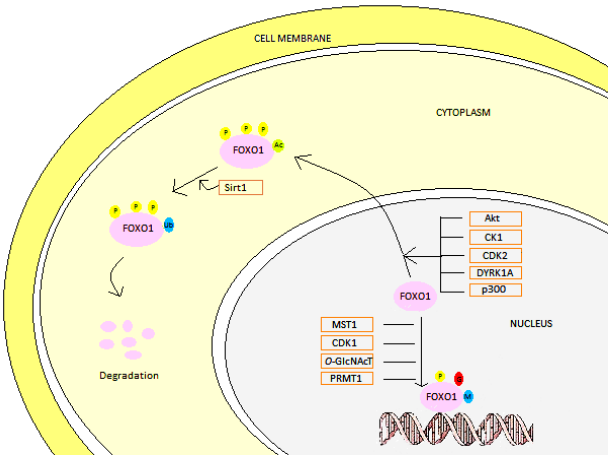

Fig 2. Mechanistic description of translocation of FOXO1. The glycosylation by OGT methylation by protein arginine methyl transferase 1 (PRMT1), and phosphorylation suing CDK1 and MST1 promotes FOXO1 binding with DNA and enhance nuclear accumulation. Acetylation by p300 and phosphorylation of FOXO1 using different kinases like Akt, CK1, CDK2, DYRK1A reduces DNA binding affininty of FOXO1 and enhance cytoplasm localization where FOXO1 undergoes degradation upon deacetylation by Sirt1.

immune responses. The interplay between phosphorylation and glycosylation regulate sub-cellular localization of FOXO1, and affect processes such as apoptosis, gluconeogenesis and lipogenesis. Similarly if methylation of FOXO1 occurs in vicinity of acetylation and phosphorylation, it may lead to deacetylation of FOXO1, and promote binding between DNA and FOXO1. Our results also suggest that two residues K207 and K270 are equally susceptible to both acetylation and methylation, and thereby directly inhibit the effect of each other. Methylation of FOXO1 inhibits phosphorylation, and acetylation, and may promote glycosylation and enhance FOXO1 transcriptional activity. The internal interplay between different PTMs provides multifunctionality to the proteins. This multifunctional character regulates the transcriptional activity of various genes and has a crucial role in pathological conditions.

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