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14. ABSTRACT

Purpose: The purpose of this study is to determine the mechanisms by which the posttranslational SUMO modification regulates the activity of the forkhead box protein A1 (Foxa1). Major findings: We have demonstrated the sumoylation of Foxa1 in several breast cancer cell lines. Analysis of the Foxa1 protein sequence identified two potential sumoylation sites. Lysine to arginine substitution of the conserved lysine (K6) abolished Foxa1 sumoylation suggesting that the K6 is the primary sumoylation site. In contrast to the related forkhead box protein A2 (Foxa2) in which the K6R mutation induced protein destabilization, mutation of the conserved K6 sumoylation site did not strongly affect the stability of the Foxa1 protein. In transfection experiments, Foxa1 induced activation of the p27Kip1 promoter activity was downregulated by SUMO-1 demonstrating that SUMO-1 negatively regulates Foxa1 activity. On the contrary, the nonsumoylatable mutant of Foxa1 (Foxa1K6R) activated the p27Kip1 promoter to a lower extent compared with the wild type Foxa1 suggesting that the K6 and its modification/s are required for the transcriptional activity of Foxa1. Together, our results show that Foxa1 is modified by sumoylation on lysine, K6, and the SUMO modification of Foxa1 modulates the activity of Foxa1 on its target genes in breast cancer cells.

15. SUBJECT TERMS
Estrogens, Estrogen receptor, Foxa1, SUMO-1, Ubc9, PIAS1, Sumoylation

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Introduction:

Estradiol (E2) and its receptor, estrogen receptor (ER) alpha, regulate the growth potential of breast cancer cells. Recent genome wide chromatin immunoprecipitation (ChIP) and bioinformatics studies have shown that the specificity of ER target genes is determined by the forkhead box transcription factor A1 (Foxa1), which binds in close proximity to ER binding sites (1,2,5,6). Foxa1 was required for binding of ER, regulation of ER target genes and estrogen-induced reentry of breast cancer cells into cell cycle (2,5). Recently, the breast cancer susceptibility gene, BRCA1, was shown to stabilize Foxa1 suggesting that Foxa1 protein is regulated posttranslationally through stabilization and destabilization mechanisms (8). In the course of our studies on pancreatic islet gene regulation by the related forkhead box protein A2 (Foxa2), we have found that Foxa2 is sumoylated on lysine K6. Abolishing sumoylation by targeted mutagenesis of the Sumo acceptor lysine or by overexpression of the dominant negative sumo-conjugating enzyme, Ubc9, resulted in destabilization and loss of Foxa2 protein expression. In nude mice tumorigenicity assays, Ubc9, which is upregulated in ovarian cancers, promoted, while dominant negative Ubc9 interfered with the growth of MCF7 breast cancer cells (7). Foxa1 is highly similar to Foxa2 and the sumoylation sites are conserved. Based on these observations we have hypothesized that Foxa1 is sumoylated and the SUMO modification of Foxa1 plays an important role in determining the tumorigenic potential of breast cancer cells.

Body:

Task 1. As proposed under the objective 1 of our grant proposal, we have examined whether Foxa1 is sumoylated in breast cancer cells by immunoprecipitation (IP) and western blotting analysis. Equal amounts of cell lysates prepared from ER positive, Foxa1 expressing MCF7 breast cancer cells were IPd with SUMO-1, SUMO-2/3 antibodies coupled to agarose beads. Since SUMO-2 and SUMO-3 antibodies cross-react with each other due to strong sequence homology (98% aminoacid identity between mature SUMO-2 and SUMO-3) (3), antibodies that react with both SUMO-2 and SUMO-3 were used for IPs. IP reactions with normal mouse antibody coupled agarose beads served as a negative control. The IPs were resolved and analyzed by western blotting with Foxa1 antibodies. As shown in figure 1, IP with SUMO-1 antibodies resulted in a doublet of Foxa1 bands (lane 1, indicated by arrows). The lower band of the doublets was the predominant band in SUMO-2/3 IPs (figure 1, lane 2). Further, an additional band (indicated by arrowhead) and a slow migrating smear (indicated by bracket) were apparent in SUMO-2/3 antibody IPs. Currently, we do not know the identity of the additional Foxa1 antibody reactive band and the smear in SUMO-2/3 IPs. The additional band could represent Foxa1 sumoylated at multiple sites by SUMO-2/3. The high molecular weight smear in SUMO-2/3 IP could have resulted from the unique ability of SUMO-2/3 but not SUMO-1 to undergo poly-sumoylation as has been suggested earlier for other SUMO-2/3 substrates (3). The results of these experiments show that the endogenous Foxa1 expressed in MCF7 breast cancer cells undergoes sumoylation by SUMO-1 and SUMO-2/3. We did not examine the sumoylation status of Foxa1 in MDA-MB-231 cells as these cells do not express endogenous Foxa1 (9).

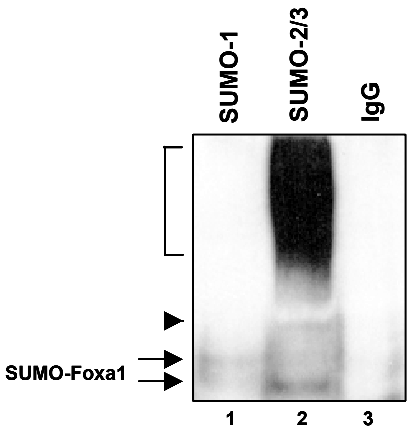

Figure 1. Foxa1 is sumoylated in MCF7 breast cancer cells. One mg of MCF7 cell lysates were immunoprecipitated using 4 μg of SUMO-1 (lane 1), SUMO-2/3 (lane 2) and normal mouse antibody (lane 3) coupled agarose beads. The IPs were extensively washed, resolved on 8% polyacrylamide-SDS gel and analyzed by western blotting with Foxa1 antibody. Sumoylated Foxa1 doublets are shown by arrows. Additional Foxa1 antibody reactive band and a high molecular weight smear are indicated by an arrowhead and a bracket.

To determine if Foxa1 is preferentially modified by a particular SUMO, we transfected MCF7 cells with expression vectors expressing HA epitope tagged Foxa1 along with or without FLAG epitope tagged SUMO-1, SUMO-2 or SUMO-3. Forty eight hours following transfection, cells were lysed and analyzed by IP with mouse HA antibody followed by western blotting with rabbit HA antibody. As shown in figure 2 (upper panels in lanes 2,3,5,6,8,9) IPd Foxa1 resolved as a doublet (indicated by arrow). Cotransfection of SUMO-1, -2 or -3 expression vectors resulted in the appearance of slow migrating sumoylated Foxa1 (figure 2, upper panels, lanes 3,6,9, indicated by arrowhead). The sumoylated bands lighted up specifically when the blots were stripped and reprobed with the FLAG antibody, which recognizes the FLAG epitope on transfected SUMO-1, -2 and -3 confirming that these slow migrating bands correspond to sumoylated Foxa1 (indicated by asterisk in figure 2, lower panels, lanes 3,6,9). Foxa1 was strongly sumoylated by SUMO-1 compared to SUMO-2 and SUMO-3 (figure 2, upper panels, lanes 3,6,9). Further, SUMO-1 sumoylated Foxa1 migrated as a doublet while SUMO-2 and SUMO-3 sumoylated Foxa1 migrated as single bands. Together, these results show that while Foxa1 is sumoylated by all three SUMOs, Foxa1 is a preferred substrate for sumoylation by SUMO-1.

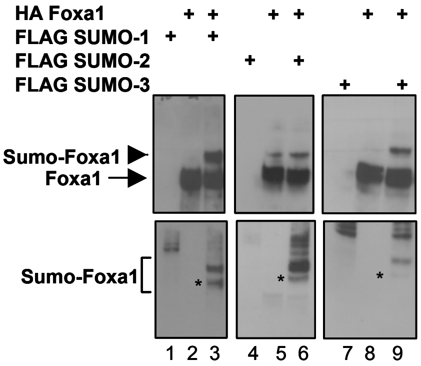

Figure 2. Foxa1 is sumoylated in MCF7 breast cancer cells. Subconfluent MCF7 cells were transfected with 1 μg of expression vectors for HA epitope tagged Foxa1 (lanes 2,3,5,6,8,9) with FLAG epitope tagged SUMO-1 (lane 3), FLAG epitope tagged SUMO-2 (lane 6) and FLAG epitope tagged SUMO-3 (lane 9). Cells corresponding to lanes 1, 4, and 7 were transfected with FLAG epitope tagged SUMO-1, SUMO-2 and SUMO-3, respectively. Total amount of transfected DNA was adjusted to 2 μg using pCDNA3 empty vector. Forty eight hours following transfection, equal amounts of cell lysates were IPd with mouse HA antibody and analyzed by western blotting with rabbit HA antibody (upper panels). Unsumoylated Foxa1 is indicated by an arrow and the sumoylated Foxa1 by arrow head in the upper panel. The blots were stripped and reprobed with rabbit FLAG antibody and shown in lower panels. Asterisks in the lower panel indicates the bands that correspond to the sumoylated Foxa1 bands shown in the upper panels. Additional sumoylated bands are shown by a bracket in the lower panel.

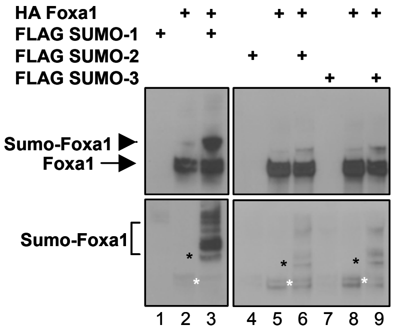

Figure 3. Foxa1 is sumoylated in MDA-MB-231 breast cancer cells. Subconfluent MDA-MB-231cells were transfected with 1 μg of expression vectors for HA epitope tagged Foxa1 (lanes 2,3,5,6,8,9) with FLAG epitope tagged SUMO-1 (lane 3), FLAG epitope tagged SUMO-2 (lane 6) and FLAG epitope tagged SUMO-3 (lane 9). Cells corresponding to lanes 1, 4, and 7 were transfected with FLAG epitope tagged SUMO-1, SUMO-2 and SUMO-3, respectively. Total amount of transfected DNA was adjusted to 2 μg using pCDNA3 empty vector. Forty eight hours following transfection, equal amounts of cell lysates were IPd with mouse HA antibody and analyzed by western blotting with rabbit HA antibody (upper panels). Unsumoylated Foxa1 is indicated by an arrow and the sumoylated Foxa1 by arrow head in the upper panel. The blots were stripped and reprobed with rabbit FLAG antibody and shown in lower panels. Black asterisks in the lower panel indicates the bands that correspond to the sumoylated Foxa1 bands shown in the upper panels. Additional sumoylated bands are shown by a bracket in the lower panel. Signals from the incompletely stripped Foxa1 band is shown by white asterisks in the lower panels.

We have also examined whether Foxa1 is sumoylated in ER negative MDA-MB-231 cells by transfecting these cells with expression vectors expressing HA epitope tagged Foxa1 with or without FLAG epitope tagged SUMO-1, SUMO-2 or SUMO-3. Forty eight hours following transfection, cells were lysed and analyzed by IP with mouse HA antibody followed by western blotting with rabbit HA antibody. As seen in MCF7 cells, transfected Foxa1 resolved as a doublet in MDA-MB-231 cells. Transfected Foxa1 was sumoylated in these cells by SUMO-1, SUMO-2 and SUMO-3 (figure 3, upper panels, lanes 3,6,9). Similar to MCF7 cells, Foxa1 was preferentially sumoylated by SUMO-1 in MDA-MB-231 cells (figure 3, upper panels, compare lanes 3,6,9). Our results suggest that although MDA-MB-231 cells do not express endogenous Foxa1, they do sumoylate the transfected Foxa1 in essentially similar manner to that of the Foxa1 positive MCF7 cells.

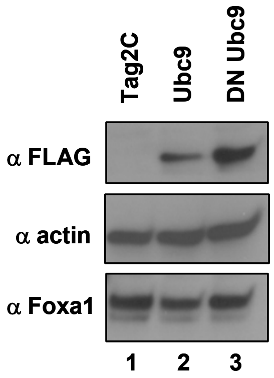

Figure 4. Stable expression of DN Ubc9 does not affect Foxa1 steady state levels. MCF7 cells were transfected with Tag2C empty vector (lane 1), FLAG epitope fused wild type Ubc9 (lane 2) or FLAG epitope tag fused DN Ubc9 (cysteine 93 mutated to serine) and selected for growth in 1 mg/ml G418. G418 resistant colonies were Pooled. 30 µg of total protein from the pooled clones was analyzed by western blotting with antibodies to FLAG epitope (top panel), internal standard actin (middle panel) and Foxa1 (lower panel).

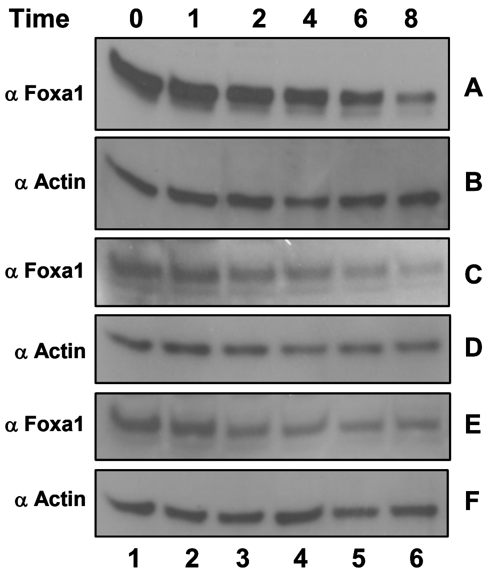

Figure 5. Foxa1 protein half life determination in MCF7 cells transfected with wild type Ubc9, DN Ubc9 or the Tag2C empty vector. Pooled G418 resistant MCF7 colonies transfected with the Tag2C empty vector (panels A,B), wild type Ubc9 (panels C,D) and DN Ubc9 (panels E,F) were treated with cycloheximide (50 µg/ml final concentration) and harvested at the indicated time points. 20 µg of total protein was analyzed by western blotting with the Foxa1 antibody (panels A, C, E) or the internal standard actin antibody (panels B,D,F). For each time point Foxa1 and the corresponding actin signals were quantitated by densitometry and normalized Foxa1 signals were used to calculate the protein half life. The experiments were repeated 3 times. A representative gel is shown.

Task 2. As proposed under the objective 2 of our grant proposal, we have generated MCF7 cells stably expressing the FLAG epitope tagged wild-type or dominant negative Ubc9 (DN Ubc9). Ubc9 is an E2-conjugating enzyme essential for sumoylation of SUMO substrates and DN Ubc9 has been shown to downregulate sumoylation of SUMO substrates (4). By using Quick Change mutagenesis system (Stratagene Inc, La Jolla, CA), we have generated a DN Ubc9 by mutating the conserved cysteine 93 to serine. Wild type Ubc9 and DN Ubc9 were cloned into G418 selectable, FLAG epitope containing pCMVTag vectors. MCF7 cells transfected with Ubc9, DN Ubc9 or the pCMVTagC empty vector were selected for growth in G418 containing medium (1 mg/ml). To minimize the variation among cell lines, G418 resistant colonies were pooled and the expression of Ubc9 and DN Ubc9 in these pools were confirmed by western blotting analysis (figure 4, top panel). Wild type Ubc9 expression was lower in these pools compared with the DN Ubc9. To determine whether the steady state levels of Foxa1 is altered by expression of DN Ubc9, we analyzed equal amounts of cell lysates from Ubc9, DN Ubc9 and vector transfected control cells by western blotting with Foxa1 antibody. Contrary to our expectation, Foxa1 levels (normalized to actin internal standard) were not downregulated by DN Ubc9 expression (figure 4, bottom panel). We further examined if the stability of Foxa1 protein is altered in these stable pools by performing cycloheximide (CHX) chase experiments. Pooled clones were treated with CHX (50 µg/ml final concentration) and harvested at 0, 1, 2, 4, 6 and 8-hour time points and analyzed by western blotting with the Foxa1 antibody or the internal standard actin antibody. A representative experiment is shown in the figure 5. Foxa1 and the corresponding actin signals for each time point were quantitated by densitometry. Normalized Foxa1 signals from 3 experiments were used to calculate the half-life of Foxa1 protein. Half-life of Foxa1 protein was approximately 3 hours 45 minutes in Tag2C, Ubc9 and DN Ubc9 expressing cells. Together, these experiments indicate that modulating the activity of SUMO ubiquitin conjugating enzyme does not affect Foxa1 stability.

We have also used an alternate approach to examine whether sumoylation affects the stability of Foxa1 protein. Foxa1 contains 2 potential sumoylation sites: lysine 6 and lysine 265 (figure 6, panel A). We mutated the conserved lysine (K) 6 into arginine (R). HA epitope tagged Foxa1 (figure 6, lanes 1,2) or Foxa1K6R (figure 6, lanes 3,4) were transfected with out (lanes 1,3) or with (lanes 2,4) FLAG epitope tagged SUMO-1 into MCF7 cells (figure 6B, top panel) and MDA-MB-231 cells (figure 6B, bottom panel). Equal amounts of cell lysates were IPd with mouse HA antibody and analyzed by western blotting with rabbit HA antibody. As shown in figure 6B. K6R mutation abolished sumoylation of Foxa1 in both MCF7 cells (top panel, figure 6B) and MDA-MB-231 cells (bottom panel, figure 6B). Despite loss if sumoylation, steady state levels of Foxa1 was not altered in both MCF7 and MDA-MB-231 cells suggesting that Foxa1 protein expression levels are not affected by the sumoylation.

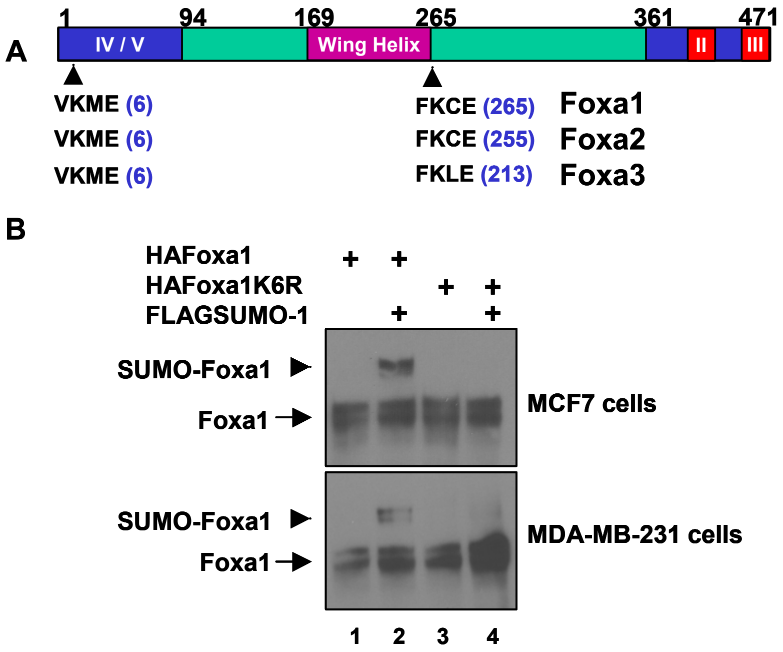

Figure 6. Panel A. Diagrammatic representation of Foxa1 protein. The activation domains II, III, IV and V and the DNA binding winged helix forkhead box of human Foxa1 protein are shown. The numbers above the diagram correspond to aminoacid coordinates of human Foxa1. Potential sumoylation sites (denoted by arrowheads) and sequences are shown below the diagram. Numbers in parenthesis next to the aminoacid sequences correspond to the potential sumoylation sites for human Foxa1, Foxa2 and Foxa3. **Panel B . Foxa1 protein stability is not affected by sumoylation. Top panel.** MCF7 breast cancer cells were transfected with HA epitope tagged Foxa1 (lanes 1,2) or HA epitope tagged K6R mutated Foxa1(lanes 3,4), with out (lanes 1,3) or with (lanes 2,4) FLAG epitope tagged SUMO-1. 48 hours following transfections, equal amounts of cell lysates were immunoprecipitated with the mouse HA antibody and analyzed by blotting with rabbit HA antibody. Non sumoylated Foxa1 is indicated by arrow. Sumoylated Foxa1is indicated by arrowhead. **Bottom panel.** Descriptions similar to the top panel except that MDA-MB231 cells were used for the experiments.

Task 3. As proposed under the **objective 3**, we attempted to strongly and stably express Foxa1 in MCF7 cells. Although Foxa1 can be expressed transiently in MCF7 and MDA-MB-231 cells, stable expression from CMV promoter resulted in loss of clonal expansion of cells and eventual loss of cell lines. Therefore stable MCF7 cell lines expressing tetracycline inducible Foxa1 are being developed. Based on a previously published work showing activation of $p27^{Kip1}$ promoter by Foxa1 (8), we reasoned that strong activation of the endogenous $p27^{Kip1}$ cell cycle inhibitor may be responsible at least in part for our inability to derive stable MCF7 cell lines expressing constitutively high levels of Foxa1. Therefore we examined the effect of wild type and sumoylation deficient mutant of Foxa1 and SUMO-1 on the activity of $p27^{Kip1}$ promoter. Foxa1 induced activation of the $p27^{Kip1}$ promoter activity was downregulated by SUMO-1 demonstrating that SUMO-1 negatively regulates Foxa1 activity. On the contrary, the nonsumoylatable mutant of Foxa1 (Foxa1K6R) activated the $p27^{Kip1}$ promoter to a lower extent compared with the wild type Foxa1 suggesting that the K6 and its modification/s are required for the transcriptional activity of Foxa1 (figure 7).

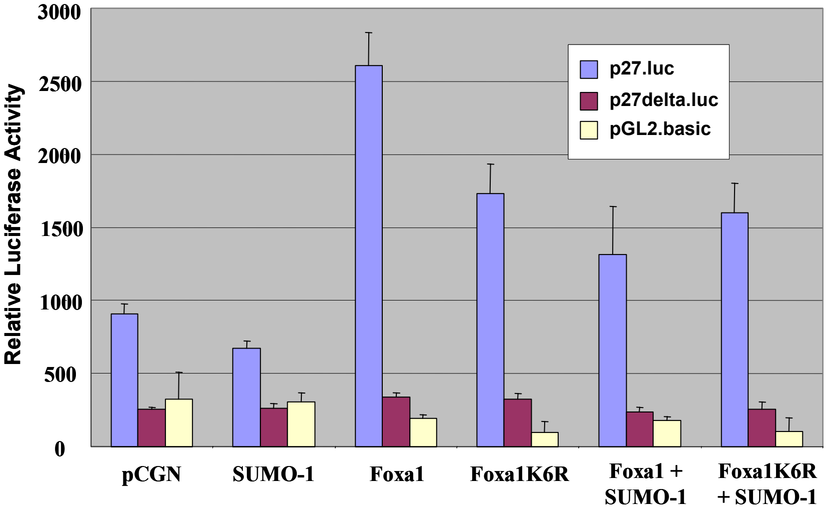

Figure 7. Sumoylation modulates the Foxa1 activation of the p27^{Kip1} promoter. Subconfluent MCF7 cells were cotransfected with the p27Kip1 promoter luciferase reporter (-2002 to +154) along with the pCGN empty vector or SUMO-1 expression vector or vectors encoding Foxa1 or Foxa1K6R mutant as indicated. pGL2 basic reporter with no promoter-enhancer elements or the p27Kip1 promoter containing the promoter proximal 45 bp sequences were used as negative controls. 48 hours following transfection, cells were harvested and luciferase activity analyzed. Results are shown as mean +/- standard deviation from two independent experiments performed in duplicates.

The animal experiments proposed under **task 3** will be performed once conditional Foxa1 overexpressing MCF7 cell lines are derived.

Task 4. Due to our inability to obtain Foxa1 overexpressing MCF7 cells, we have not been able to accomplish this task

Key research accomplishments:

1. Demonstration of sumoylation of endogenous Foxa1 in MCF7.
2. Demonstrating that transfected Foxa1 is sumoylated by SUMO-1, SUMO-2 and SUMO-3 in both MCF7 and MDA-MB-231 cells.
3. Demonstration of preferential sumoylation of Foxa1 by SUMO-1 in MCF7 and MDA-MB-231 breast cancer cells.
4. Mapping the sumoylation sites and determining that sumoylation does not affect the stability of Foxa1 in MCF7 and MDA-MB-231 cells.
5. Demosntrating that strong stable expression of Foxa1 affects clonal expansion and causes loss of cell lines.
6. Demonstrating that sumoylation modulates the Foxa1 mediated activation of its target genes such as p27^{Kip1}.

Reportable outcomes:

This work is being submitted for presentation at the upcoming Era of Hope meeting. This work will be submitted for publication in a reputed journal that publishes cancer related work once the work is completed.

Conclusions:

Together, our results show that Foxa1 is modified by sumoylation on lysine, K6, and the SUMO modification of Foxa1 modulates the activity of Foxa1 on its target gene promoters such as, p27^{Kip1}. Further, our work suggests that the K6 residue of Foxa1 may be a target for multiple competing modifications and the nature of the modification/s determines the activity of Foxa1 towards its target genes.

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Appendices: None