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Cross-talk between sumoylation and phosphorylation in mouse spermatocytes

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Abstract

The meiotic G2/M1 transition is mostly regulated by posttranslational modifications, however, the cross-talk between different posttranslational modifications is not well-understood, especially in spermatocytes. Sumoylation has emerged as a critical regulatory event in several developmental processes, including reproduction. In mouse oocytes, inhibition of sumoylation caused various meiotic defects and led to aneuploidy. However, the role of sumoylation in male reproduction has only begun to be elucidated. Given the important role of several SUMO targets (including kinases) in meiosis, in this study, the role of sumoylation was addressed by monitoring the G2/M1 transition in pachytene spermatocytes *in vitro* upon inhibition of sumoylation. Furthermore, to better understand the cross-talk between sumoylation and phosphorylation, the activity of several kinases implicated in meiotic progression was also assessed upon down-regulation of sumoylation. The results of the analysis demonstrate that inhibition of sumoylation with ginkgolic acid (GA) arrests the G2/M1 transition in mouse spermatocytes preventing chromosome condensation and disassembling of the synaptonemal complex. Our results revealed that the activity of PLK1 and the Aurora kinases increased during the G2/M1 meiotic transition, but was negatively regulated by the inhibition of sumoylation. In the same experiment, the activity of c-Abl, the ERKs, and AKT were not affected or increased after GA treatment. Both the AURKs and PLK1 appear to be “at the right place, at the right time” to at least, in part, explain the meiotic arrest obtained in the spermatocyte culture.

Keywords

meiosis; phosphorylation; sumoylation; ginkgolic acid; AURKs; PLK1

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Introduction

Successful progression through the G2/M1 transition in spermatocytes is a prerequisite for the formation of normal, genetically balanced gametes. Errors in meiotic recombination and chromosome segregation are the major causes of meiotic nondisjunction and aneuploidy [1]. However, the molecular mechanisms responsible for these meiotic errors are not well-understood, especially in males. Knockout mouse models arrested at the G2/M1 transition, in addition to the development of an *in vitro* system where pachytene spermatocytes have been induced to undergo G2/M1 transition upon treatment with okadaic acid (OA, an inhibitor of phosphatases PP1 and PP2A), have identified several important regulators of the process. For example, mice with a deletion of Cyclin A1 (CCNA1) or spermatocyte-specific deletion of cyclin dependent kinase 1 (CDK1) exhibit meiotic arrest during the exit from the meiotic prophase [2] [3]. In a similar manner, mice with meiotic expression of an inactive isoform of AURKB display abnormalities during the exit from meiosis I [4]. Events in spermatocytes during the OA-induced G2/M1 transition *in vitro* closely mimic the ones observed *in vivo*. The Initiation of desynapsis is hallmarked by the removal of the central element protein (SYCP1) of the synaptonemal complex (SC) and precedes phosphorylation of histone H3 on Serine 10 (H3SerPh). These events are followed by the re-localization of the lateral element protein of the SC (SYCP3) to the centrosomes, and the formation of condensed bivalents. Although the translation of specific proteins during the pachytene stage is a prerequisite for the successful completion of meiosis, inhibition of protein synthesis in spermatocytes at the time of G2/M1 transition does not affect the exit from meiotic prophase *in vitro* [5]. Therefore, the G2/M1 transition is mostly regulated by posttranslational modifications. In support of this finding, the inhibition of global tyrosine phosphorylation or the activity of specific kinases causes meiotic arrests at different stages during the OA-induced G2/M1 progression [6, 7]. For example, CDK inhibitor Butyrolactone (BLI) does not affect desynapsis or H3SerPh but does inhibit the OA-induced re-localization of SYCP3 and condensation of bivalents [8]; Interestingly, CDK inhibition also inhibits the activity of mitogen-activated protein kinases (MAPKs) through an unknown mechanism [9]. ZM447439 (ZM), an inhibitor of AURKs, did not affect initiation of desynapsis but inhibited H3SerPh (which is a direct target of AURKB) [7]. Inhibition of PLK1 with dihydropteridinone BI 2536 fully inhibited the first step of desynapsis (the removal of SYC1) and affected H3 phosphorylation to a certain degree [6]. Notably, general tyrosine kinase inhibitor completely abolishes desynapsis and chromosome condensation [5]. In addition to kinase activity, the activity of topoisomerases (TOPs, enzymes unwinding DNA) is also required for G2/M1 progression. Teniposide and ICRF-193, inhibitors of TOP2, dramatically affect the condensation of chromosomes [8].

Sumoylation is yet another type of posttranslational modification by Small Ubiquitin-like Modifiers or SUMO proteins that has been identified as an important regulatory event in several cellular processes including cell cycle progression [10–14]. Covalent conjugation of SUMO to the target protein happens through the action of SUMO activating enzyme (E1, a heterodimer of SAE1-SAE2), SUMO-conjugating enzyme UBC9 (E2), and SUMO ligases (E3). Sumoylation is reversed through the action of SENPs, which cleave the isopeptide bond between SUMO and its substrate. Four SUMO paralogs have been identified: SUMO1,

2, 3 (often termed SUMO2/3 because of their 95% sequence identity), and 4. While SUMO1, 2, and 3 are abundantly expressed in different tissues, SUMO4 is restricted to the kidneys and lymphatic tissues [15–17]. SUMO1-knockout mice show no phenotypic consequences because the protein's function is compensated for by SUMO2 and SUMO3 [18]. SUMO2 is apparently the major SUMO isoform present during embryonic development, and as a result, SUMO2-knockout mice show early embryonic lethality [19]. In a similar manner, UBC9 (the only SUMO-conjugating enzyme)-knockout mice show early embryonic lethality with severe disruptions in mitosis; a finding that supports the indispensable role of sumoylation in cell cycle progression [20]. In mouse oocytes, sumoylation plays a crucial role in spindle organization in addition to chromosome congression and segregation. Inhibition of sumoylation by SUMO1 or UBC9 with a specific antibody or their depletion by specific si-RNA microinjection in oocytes caused defective spindle organization, misaligned chromosomes, and led to aneuploidy. In a similar manner, over-expression of SENP-2, a SUMO-specific isopeptidase, led to defects in MII spindle organization in mature eggs [21, 22]. However, the role of sumoylation in male reproduction has only begun to be elucidated. We and others have localized SUMO proteins in germ and somatic testicular cells and have obtained evidence implicating sumoylation in different aspects of normal and impaired spermatogenesis. We have also identified sumoylated targets in purified mouse spermatocytes and spermatids and in human sperm. In mouse spermatocytes, numerous proteins have been identified as SUMO targets including those regulating transcription, chromatin dynamics, and other processes. Sumoylation of several targets with potentially important roles during meiosis (such as CDK1, TOP2, RNAP II, MILI, DDX4, MDC1, KAP1, and TDP-43) were further supported by co-immunoprecipitation, co-localization, and *in vitro* sumoylation studies [23–28] [29]. SYCP1 and SYCP2 have also been co-immunoprecipitated with SUMO from testicular lysate, as shown by another group [23]. Interestingly, some kinases have been identified by our screen as SUMO targets [29]. This finding is consistent with growing evidence that phosphorylation and sumoylation interact at multiple levels. A phosphorylation-dependent motif has been identified [30] and inhibition of sumoylation in somatic cells by a sumoylation inhibitor (ginkgolic acid, GA) significantly affected tyrosine phosphorylation (PhosphoTyr) of multiple proteins [31].

Overall, a cross-talk between different post-translation modifications during meiotic prophase and the G2/M transition is not well-understood, especially in spermatocytes. Given the important role of several SUMO targets in meiosis, in this study, the role of sumoylation was addressed by monitoring the G2/M1 transition in pachytene spermatocytes *in vitro* upon inhibition of sumoylation. Furthermore, to better understand a cross-talk between sumoylation and phosphorylation, the activity of several kinases implicated in meiotic progression was also assessed upon down-regulated sumoylation.

Materials and methods

Short-term culture of mouse spermatocytes; okadaic and ginkgolic acid treatment

C57BL/6NCrl mice were purchased from Charles River (Kingston, NY). The Animal Committee of Albert Einstein College of Medicine, Yeshiva University approved all animal

protocols. A spermatocyte-enriched fraction was prepared as described in our recent publication. A flow-cytometry and microscopic analysis to confirm fraction purity was also performed as previously described by our group [29]. Spermatocytes were cultured according to [32]. Spermatocyte-enriched fractions were pooled together, washed three times by centrifugation at 500 g for 7 min, and resuspended in minimal essential medium (MEM) (Sigma-Aldrich, M0894) supplemented with 0.29% (v/v) DL-lactic acid, 5% (v/v) FBS, 5.9 mg/ml HEPES, 0.05 mg/ml streptomycin sulfate (Life Technologies, 11860-038), and 0.075 mg/ml penicillin G (Sigma-Aldrich, P3032) to the final concentration of 2.5×10^6 cells/ml. Next, 986.7 μ l of cell suspension was added to each well of a 4-well dish (Thermo Scientific, Rockford, IL), and the cells were incubated at 32°C with 5% CO₂ for 10 hours (after STA-PUT separation) or were used the same day after differential plating. The results of the experiments were similar with the both types of spermatocyte isolation, but the differential plating procedure shortened the time of the experiment by one day. Following the incubation, 1 μ l of freshly prepared ginkgolic acid (GA, a specific inhibitor of sumoylation that blocks formation of the E1-SUMO intermediate [33]) or 1 μ l of 100% DMSO was added to the experimental or control wells, respectively. The final concentration of GA was 10–50 μ M. After one hour, G2/M transition was induced by the addition of 13.3 μ l of 300 μ M okadaic acid (OA, a phosphatase inhibitor [7, 34]) for an additional 4 hours. Following treatment, 100 μ l of cell suspension from each well was centrifuged at 5000 rpm (Eppendorf, 5415 C, 5 min, 4°C) and resuspended in 20 μ l of a 2% PFA + 0.03% SDS solution. To this, 20 μ l of 0.4% Photoflo solution (Kodak Professional, #74257, Hatfield, PA) was added. For chromosome spreads, 2 μ l of well-mixed cell suspension was pipetted onto each well of a Shandon™ multi-spot slide (Thermo Scientific, 9991090) and gently spread in a circular motion over the well using a tip. After a brief air drying of the cells (5–10 min), the slides were washed in a series of solutions in Coplin jars: a. 2% PFA + 0.03% SDS for 3 min; b. 2% PFA for 3 min; c. 0.4% Photoflo for 3 $\times$ 1 min. The slides were then air dried for 1 hour before being subjected to IF or storage at –20°C. In some experiments, the remaining 900 μ l of cell suspension from each well was used for preparation of cell lysate. Whole cell protein lysates were prepared as previously described, using the whole cell extraction kit and protease inhibitor from Millipore (2910, Billerica MA) complemented with 2.5 mg/ml of N-ethylmaleimide (NEM (a de-sumoylation inhibitor), E3876-100G, Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitors (88667, Thermo Fisher Scientific, Rockford, IL). IF was performed as previously described. Information regarding the source, vendor, and dilution of the antibodies used in this study is summarized in Table 1.

Results and Discussion

Given the important roles of several SUMO targets in meiosis, we sought to examine whether sumoylation is important for the meiotic prophase I to metaphase I transition. The effect of different concentrations of a sumoylation inhibitor (GA) upon sumoylation in spermatocytes was first tested using western blot analysis. Compared with HEK cells, in which sumoylation was inhibited only by a high concentration of GA (e.g., 100–200 μ M for 6 hours (in accordance with previous reports in somatic cells [33], spermatocytes were highly sensitive to GA and showed a significant inhibition of sumoylation at 20 μ M after just

1 hour and at 10 μ M after 3 hours (Suppl. Fig. 1A, B₁, and B₂). Doses of 40 and 50 μ M also caused significant cell death and as such were not used for further experiments.

The G2/M1 transition was induced *in vitro* by treating the highly purified pachytene spermatocyte fraction (Fig. 1A) with the phosphatase inhibitor okadaic acid (OA) with and without a preceding addition of 30 μ M GA [7, 34]. H3Ser10 phosphorylation was used as a marker of chromosome condensation initiated at the diplotene stage, and anti-SYCP3 antibody was used as a marker of the lateral element of the synaptonemal complex. The results of the analysis demonstrate that inhibition of sumoylation with GA arrests the G2/M1 transition in mouse spermatocytes. As can be seen from Fig. 1 (B, C), in the control culture (–GA, B), treatment with OA induced condensation of chromosomes accompanied by massive H3Ser10 phosphorylation (red) and full disassembly of the SC (green) as previously reported [8, 34, 35]. In contrast, the addition of GA (+GA, C) prevented chromosome condensation and disassembling of the SC. The percentage of cells arrested at the G2/M1 transition upon treatment with GA increased in a concentration-dependent manner Fig. 2 (A). Similar to the results in somatic cells, inhibition of sumoylation significantly affected the global phosphorylation (PhosphoTyr; Fig. 2B₁). In a similar manner to the chemical inhibition, specific inactivation of sumoylation using UBC9 (SUMO-conjugating enzyme) si-RNA caused significant changes in PhosphoTyr of the GC-1 spermatogonia cell line (Fig. 2B₂). Interestingly, the timing of the meiotic arrest was similar to the one observed upon inhibition of tyrosine phosphorylation [5].

The role of tyrosine phosphorylation and its regulation by specific tyrosine kinases or phosphatases is not well understood in spermatocytes. One exception is a study of c-Abl kinase, which was shown to be highly expressed in spermatocytes and required for normal spermatogenesis [36]. Based on these data, we used an antibody against an activated form of c-Abl to examine changes in its activity upon inhibition of sumoylation. Our data suggest that addition of OA did not change the level of activated c-Abl, and inhibition of sumoylation did not inhibit (but rather slightly activated) c-Abl (Fig. 3, Ph-Abl), suggesting that other unknown tyrosine kinases or phosphatases contributed to significant changes (mostly decreases) in the level of global tyrosine phosphorylation observed after GA treatment (Fig. 2. B₁).

Global changes in serine/threonine (Ser/Thr) phosphorylation were less prominent, probably due to the poor quality of available antibodies (not shown). Therefore, we used specific antibodies against either activating (PLK1, AUR, ERK, AKT) or inhibitory (CDC2) phosphorylation on several Ser/Thr kinases implicated in the regulation of meiosis. Our results revealed that the activity of PLK1 and the Aurora kinases increased during the G2/M1 meiotic transition (+OA), but was negatively regulated by the inhibition of sumoylation (+30 μ M of GA). In the same experiment, the activity of the ERKs and AKT were not affected or increased after GA treatment (Fig. 3). The results for the CDC2 experiment were inconclusive because of low signal intensity (Fig. 3); however, our recently published data in cell lines suggests that inhibition of sumoylation by different means activates CDC2, so such activation would probably not be a contributing factor to the meiotic arrest [37]. Both the AURKs and PLK1 appear to be “at the right place, at the right time” to at least, in part, explain the meiotic arrest obtained in the spermatocyte culture.

AURKB is responsible for phosphorylation of H3 on Ser10 [8] and PLK1 is responsible for the disassembling of the SC [6]. As discussed above, an inhibitor of the AURKs prevented H3Ser phosphorylation during the G2/M1 transition in spermatocytes [7]. In a similar manner, mice with expression of an inactive isoform of AURKB display meiotic abnormalities [4]. Inhibition of PLK1 activity fully inhibited the first step of desynapsis (the removal of SYC1) and affected H3 phosphorylation to certain degree [6]. In addition to regulating kinase activity, sumoylation may regulate other proteins required for meiotic progression such as TOP2a (involved in meiotic chromosome condensation), or directly affect the assembly of the SC through modification of SC proteins. Indeed, the fact that SYCPs were found to be sumoylated [23] and interact with UBC9 (the SUMO-conjugating enzyme) [38] supports an important role for SUMO at the time of the observed spermatocyte arrest. Further studies are required to understand how sumoylation regulates events responsible for the disassembly of the SC.

In other studies, La Salle et al. [39] showed that upon the transition from pachytene to diplotene (the time when meiotic arrest occurs upon inhibition of sumoylation), both SUMO1 and SUMO2/3 remained localized to the XY body, but the centromeric signal was significantly diminished. We also failed to observe differences in the localization of SUMO1 and SUMO2/3 upon transition from pachytene to diplotene. Furthermore, western blots of OA-induced spermatocytes with and without the addition of GA showed very similar patterns for SUMO1 and SUMO2/3 (Supplementary Figure 2). It can therefore be suggested that GA-induced inhibition of SUMO-conjugating machinery affects SUMO1 and SUMO2/3 conjugation to a similar degree. However, La Salle et al. observed a different pattern of SUMO1 and SUMO2/3 in MI spermatocytes, as determined by IF with the specific antibodies used in that study. Differences in the functions of SUMO1 and SUMO2/3 and the effect of GA on MI spermatocytes should be further evaluated (this may prove to be difficult because the GA would hypothetically need to be added immediately after the disassembly of the synaptonemal complex). However, possible differences in the specificity of the antibodies used for IF and the absence of a fertility phenotype in SUMO1-knockout mice (suggesting possible overlapping functions of SUMO1 and SUMO2/3) should be taken into account.

Based on the fact that kinases were identified as SUMO targets in spermatocytes, our discussion mostly focused on kinases; however, the inhibition of sumoylation may also affect the activity of phosphatases. Nevertheless, in somatic cells, when compared with cells treated with pervanadate, a strong pan-tyrosine phosphatase inhibitor, GA-treated cells showed different patterns of changes in protein tyrosine phosphorylation [31]. These results implied that SUMO regulation of protein tyrosine phosphorylation is different from the phosphatase-dependent alteration and suggest that kinases may be the major target of SUMO. Whether these conclusions are relevant to germ cells remains to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GA	ginkgolic acid
OA	okadaic acid
SUMO	Small Ubiquitin-like Modifiers

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Highlights

- Inhibition of sumoylation arrests the G2/M1 transition in mouse spermatocytes
- Inhibition of sumoylation significantly affects global phosphorylation
- PLK1 and the AURKs are negatively regulated by inhibition of sumoylation

Figure 1. Inhibition of sumoylation blocks the G2/M1 progression in spermatocytes

A highly purified population of spermatocytes (A) was treated with the phosphatase inhibitor okadaic acid (OA) with (B) and without (C) a preceding addition of 30 μ M of a sumoylation inhibitor (ginkgolic acid, GA). Color-coding is indicated for each image group. Nuclei are stained by DAPI (blue). In a control culture, OA treatment induced massive H3Ser10 phosphorylation (which hallmarks chromosome condensation) and disassembly of the synaptonemal complex (B); In contrast, neither disassembly of the synaptonemal complex nor H3Ser10 phosphorylation were detectable in GA-treated spermatocytes (C).

Figure 2.

A) Effect of increasing concentration of sumoylation inhibitor ginkgolic acid (GA) on disassembly of synaptonemal complex and appearance of H3Ser10 phosphorylation. Fifty cells were scored for each condition and the experiments were repeated three times. Data are presented and mean $\pm$ standard deviation.

B₁) The effect of inactivation of sumoylation by ginkgolic acid (GA) on tyrosine phosphorylation in spermatocytes.

Spermatocytes were treated with 30 μ M of GA for 1 hour followed by a preparation of the whole cell lysate and western blot analysis with anti-SUMO or anti-phospho-tyrosine antibody. Anti-actin antibody was used to confirm equal loading.

B₂) The effect of specific inactivation of sumoylation on tyrosine phosphorylation in GC-1 spermatogonia cell lines.

Si-RNA mediated inactivation of UBC9 (SUMO-conjugating enzyme) decreased sumoylation and caused significant changes in tyrosine phosphorylation of the GC-1 spermatogonia cell line.

Figure 3. The effect of inhibition of sumoylation during the G2/M transition on the activity of several kinases implicated in the regulation of meiosis

A purified population of spermatocytes was treated with okadaic acid (+OA) with (+GA) and without (–GA) a preceding addition of 30 µM ginkgolic acid (GA).

Specific antibodies (Table 1) against either activating (c-Abl, PLK1, AUR, ERK, AKT) or inhibitory (CDC2) phosphorylation on several kinases were used to examine changes in their activity upon addition of OA and inhibition of sumoylation.

Table 1

Summary of the antibodies used in this study.

Antibody	Manufacturer	Catalog No.	Source	Dilution for western blot
Anti-SUMO1	Abcam (Cambridge, MA)	ab32058	Rabbit	1:500
Anti-SUMO2/3	Abcam	ab3742	Rabbit	1:500
Anti-phospho H3Ser10	Millipore (Temecula CA)	05-1336-S	Mouse	1:1000
Anti-mouse IgG	GenScript (Piscataway, NJ)	A01007	Mouse	-
Anti-rabbit IgG	GenScript	A01008	Rabbit	-
Anti-actin	Santa Cruz	sc-1615	Goat	1:1000
ECL™ anti-rabbit IgG HRP linked	GE Healthcare UK Limited (Little Chalfont, Buckinghamshire, UK)	NA934V	Donkey	1:5000
Goat anti-mouse IgG (H + L) HRP	Millipore	AP308P	Goat	1:5000
Goat anti-rat IgG-HRP	Santa Cruz (Dallas, TX)	sc-2032	Goat	1:5000
Donkey anti-goat IgG-HRP	Santa Cruz	sc-2020	Donkey	1:5000
Anti-phosphor ERK	Sigma-Aldrich, St. Louis, MO	M8159	Mouse	1:1000
Anti-phosphor AKT (S473)	Cell Signaling (Beverly, MA)	4060	Rabbit	1:2000
Anti-phosphor S6 (S235/236)	Cell Signaling	4858	Rabbit	1:2000
Anti-phospho Aurora A/B/C	Cell Signaling	2914P	Rabbit	1:1000
Anti-phospho cdc2	Cell Signaling	9111P	Rabbit	1:1000
Anti-phospho Plk (Thr210)	Santa Cruz	sc-135706	Rabbit	1:100
Anti-phosphotyrosine	Millipore	16-105	Mouse	1:1000
Phospho-c-Abl	Antibodyplus (Brookline, MA)	STJ90694	Rabbit	1:1000