IDENTIFICATION AND CHARACTERIZATION OF PHYSICALLY INTERACTING PARTNERS OF RETINOIC ACID RECEPTOR ALPHA IN SERTOLI CELLS

By

LI ZHU

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of LI ZHU find it satisfactory and recommend that it be accepted.

Kwan Hee Kim, Ph.D., Chair

Michael Skinner, Ph.D.

Nancy Magnuson, Ph.D.

John Wyrick, Ph.D.

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Abstract

by Li Zhu, Ph.D.
Washington State University
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Chair: Kwan Hee Kim

Vitamin A is essential for male reproduction as demonstrated by the sterility of the rats fed with vitamin A-deficient diet. The vitamin A signal is mediated by two families of retinoid receptors, retinoic acid receptor (RAR) and retinoid X receptor (RXR). Genetic studies have shown that RARA is critical for spermatogenesis, as indicated by the sterility of the *Rara*-null mice. RARA mediates the transcription of target genes, but it can be regulated by many factors such as posttranslational modifications and coregulators. The studies presented herein were to understand mechanisms by which modulators affect the functions of RARA. We performed a yeast two-hybrid screening to identify RARA-interacting proteins with RARA as the bait and proteins from a Sertoli cell cDNA library as the prey, and obtained 15 candidates, of which small ubiquitin-related modifier-2 (SUMO-2) and glucose-regulated protein 58 (GRp58) were further investigated.

We identified SUMO-2 modification of RARA as a novel posttranslational modification for RARA. The *at*RA ligand and the amount of SUMO-2 are two key components regulating sumoylation of RARA. SUMO-2 modification of RARA affects its stability, nuclear localization and transcriptional activity. Furthermore, we also characterized the subcellular localization and protein expression of SUMO-2/3, 95% similar to each other, in testes. We found that different RA signaling pathways modulated the expression and subcellular localization of SUMO-2/3 in testes. These studies demonstrate that the relationship among RA, RARA and SUMO-2 is finely tuned in testes, and manipulation of SUMO-2 could be an alternative strategy to control the functions of RARA.

We also demonstrated that GRp58 is a novel molecular chaperone required for nuclear localization of RARA. The *at*RA was able to mobilize both GRp58 and RARA into the nucleus, and then GRp58 accompanied RARA to the ER for degradation, before it could de-couple from RARA and recycle back to the cytoplasm. Studies with sulfhydryl modifying agents allowed us to speculate that the thiol-oxidoreductase activity of GRp58 on cysteine residues in RARA might be important for ligand binding to RARA and nuclear localization of RARA. These studies indicate manipulation of GRp58 could be an alternative mechanism to modulate the functions of RARA.

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CHAPTER 1

INTRODUCTION

INTRODUCTION

TESTIS AND SPERMATOGENESIS

The testis is a complex organ where the process of spermatogenesis, responsible for the production of sperm, takes place in males. The testis is encased in a fibrous capsule referred to as the tunica. Inside the testis, there are two compartments, the seminiferous tubule and interstitial compartments. The interstitial compartment contains Leydig cell, and the blood and lymphatics vessels (1). The Leydig cells produce testosterone which acts on the seminiferous tubules to maintain spermatogenesis (1). The seminiferous tubule compartment is bounded by myoid cells, the lymphatic endothelium and acellular elements, which together form the boundary tissue of the tubule (1). Along with the basal lamina, the myoid cells provide the structural underpinning on which the Sertoli cells and basal compartment cells of the seminiferous epithelium rest (1). The Sertoli cell provides much of the structural framework of the organization of the seminiferous epithelium (1). Adjacent Sertoli cells form tight junctions to divide the seminiferous tubules into two compartments (basal and adluminal) (2, 3). In the basal compartment, the pre-meiotic germ cells are toward the basal side and the meiotic germ cells are toward the lumen in the adluminal compartment (2, 3). The tight junctions create a barrier which separates the germ cells located in the adluminal compartment from the blood supply and allows the Sertoli cells to control the microenvironment of the germ cells (2, 3).

The process in which spermatogonia form spermatozoa is termed spermatogenesis, which occurs in the seminiferous tubule (4). During this process, the germ cell undergoes four phases of development: spermatogonial stem cell formation, mitosis (spermatogonial proliferation), meiosis in spermatocytes (chromosome recombination, reduction and division), and spermiogenesis (spermatid differentiation) (5). Spermatogenesis starts with the type A spermatogonia, which undergoes four mitotic division to produce the type B spermatogonia (6, 7). Then, the type B spermatogonia enters the last mitotic division and becomes preleptotene spermatocytes, where the initiation of meiosis take place (5). The preleptotene spermatocytes synthesize DNA to prepare for the meiotic divisions and then enter prophase I of meiosis I

(5). The cells then quickly finish meiosis I and II and divide to form four haploid cells, referred to as spermatids. These spermatids will differentiate into spermatozoa by undergoing maturation processes (spermiogenesis), which include elongation, nuclear condensation, development of flagellum, and acrosome production, before being release into the lumen (8). Spermatozoa are release into the lumen of seminiferous tubule by the process called spermiation (1). Then, propelled by the contractions of peritubular myoid cells, spermatozoa exit the testis. Once the spermatozoa leave the rete testis, they enter the epididymis through efferent ducts. In the epididymis, spermatozoa gain motility, the capacity to fertilize ovum, and are stored in the caudal epididymis until ejaculation (1).

VITAMIN A

The process of spermatogenesis is regulated by hormones, such as follicle stimulating hormone (FSH), leuteinizing hormone (LH), and testosterone, thyroid hormones, vitamin A, vitamin D, and vitamin E. Vitamin A is essential for spermatogenesis and overall survival of animals (9). When animals are fed with a vitamin A-deficient diet, they develop several abnormalities such as emaciation, atrophy of several glandular organs, eye lesions and testicular degeneration in testes (10, 11). The testicular degeneration is observed by a decrease in the size of the testis and arrested spermatogenesis, with the remaining germ cells being preleptotene spermatocytes and type A spermatogonia (12). Further studies showed that primary spermatocytes undergo apoptosis and more advanced germ cell are sloughed into the lumen of seminiferous tubules (13). Injection of retinol (alcohol from of vitamin A) to these animals reinitiates spermatogenesis from type A1 spermatogonia, essentially synchronizing spermatogenesis such that only a few stages of the spermatogenic cycle are represented in the regenerated testes (12, 14, 15).

Additionally, RA is necessary for spermatogonial maturation and proper entry of germ cells into meiotic prophase in the postnatal testes. Haneji et al showed that both retinol and RA could induce differentiation of type A spermatogonia in cultured mouse cryptorchid testes (16). This conclusion was based on the morphological identification of intermediate and type B spermatogonia after 9 days of culture with retinoids (16). Recently, RA has been shown to induce the expression of Stra8 (17). The

expression of Stra8 is essential for successful meiosis in both male and female gonads and normal spermatogenesis (18). The knockout of the *Stra8* gene blocks entry into meiosis in both embryonic ovaries and pubertal testes (17, 19, 20).

RETINOID RECEPTOR

Retinoic acid is a vitamin A metabolite, whose biological activity is mediated by retinoid receptors, composed of two families, the retinoic acid receptors (RAR) and the retinoid X receptors (RXR) (21). Each has three subtypes: alpha (RARA and RXRA), beta (RARB and RXRB) and gamma (RARG and RXRG), encoded by different genes. The retinoid receptors belong to the superfamily of nuclear receptors, which includes the classical hormone subfamily, such as glucocorticoid receptor, sex hormone receptors, and the thyroid/retinoid receptor subfamily. These nuclear receptors exhibit a highly conserved structure divided into six modular domains (A-F), on the basis of homology among other members of nuclear receptor. The A/B domain has the ligand-independent transcriptional activation function (AF-1). The highly conserved C domain is the DNA binding domain (DBD), which binds to hormone response elements on target genes. The D domain is the hinge region harboring a nuclear localization signal. The E domain is another highly conserved region, composed of the ligand-binding pocket, the dimerization domain, and the domain for ligand-dependent transcriptional activation function (AF-2) (21). RAR recognizes both all-*trans* retinoic acid and 9-*cis* retinoic acid, while RXR only recognizes 9-*cis* retinoic acid.

To modulate the transcriptional activity of retinoic acid-response target genes, retinoid receptor displays bimodal transcriptional properties: either repression or activation of target gene transcription. When genes are silent, DNA is packaged with histones to form closed chromatin structure via the recruitment of corepressors (22). The corepressors such as N-CoR and SMRT bind the unliganded retinoid receptors (23, 24). In general, the corepressors are proteins with histone deacetylase activity (HDACs) or they could recruit a protein with HDAC activity. HDACs remove acetyl groups from the N-terminal of the tail domain of the core histones to increase the interaction of the N-terminal histone tails

with nucleosomal DNA, and condense the chromatin, impeding transcription of target genes. Conversely, a conformational change of retinoid receptors, induced by the ligand binding, allows the dissociation of corepressors and promotes the recruitment of coactivators, such as SRC-1/p160 family, p300/CBP or CARM-1, leading to the activation of target genes (25, 26). The coactivators have a common feature, in that they have a highly conserved leucine-rich (LXXLL) helix for the interaction with nuclear receptor (26). Some of these coactivators such as p300/CBP can recruit histone acetyltransferase (HAT) or histone methyltransferase (HMT) to modify the condensed chromatin structure through acetylating or methylating specific lysine or arginine residues at the N-terminal tails of histones, thereby opening the chromatin. In addition, ATP-dependent chromatin remodelers (SWI/SNF) are recruited to translocate nucleosomes away from proximal promoter region (27). Once the chromatin is open, RNA polymerase II and general transcriptional factors (GTFs) are recruited to transcribe target genes to initiate transcription (28).

RETINOIC ACID RECEPTOR ALPHA

Genetic studies have shown that one of retinoid receptors, RARA, is critical for spermatogenesis, as shown by the sterility phenotype of male mice lacking *Rara* gene (29). The testes from *Rara*-null mouse have a testicular morphology roughly similar to that of the VAD testes (29, 30). Further characterization revealed a role of RARA in the synchronization of the spermatogenic cycle (31, 32). A normal proportion of the spermatogenic stages of the seminiferous epithelial cycle could not be maintained without RARA; instead, a major disorganization of germ-cell association was observed (31, 32). In addition, Doyle, et al (30) found that RARA may function in Sertoli cells to promote the survival and development of early meiotic prophase spermatocytes, whereas RARA in germ cells may function to increase the proliferation and differentiation of spermatogonia before meiotic prophase. Also of interest is that both Stra8 mRNA and protein, which is induced by RA, important for initiation of meiosis, were detected in *Rara* knockout mice (unpublished data). This indicates that RARA in germ cells must be responsible for a later phase of germ development, after the STRA8 function in the testis or there are two parallel pathways, one with STRA8 as its component and the other with RARA as its component in the

testis. Hence, it is clear that RARA is an essential component for normal spermatogenesis and it is important to understand how it is regulated in the testis if we are to unravel the role of RA in germ cells and in Sertoli cells.

POSTTRANSLATIONAL MODIFICATIONS

Posttranslational modifications such as phosphorylation and ubiquitination of nuclear receptors have proven to be important mechanisms in regulating the dynamic nuclear localization and transcriptional functions of nuclear receptors (33). RAR has been shown to be post-translationally modified by a variety of protein kinases, including PKA, PKC, CDK7, and p38MAPK (34-36), and ubiquitinated (37, 38), impacting the subcellular localization, degradation, and transcriptional activity of RARA. Our lab showed that PKA, activated by a short-term exposure of FSH, inhibited *at*RA-induced RARA nuclear localization, decreased the steady state level of RARA, and the RARE-dependent transcription in a mouse Sertoli cell line stably transfected with the FSH receptor cDNA (39). However, it is not known whether PKA induced by FSH directly phosphorylate RARA. This is currently being investigated. Meanwhile, RARA has been shown to be positively regulated by PKC and MAPK in Sertoli cells, independent of the ligand (40).

Additionally, in response to RA, RARs, as well as their coactivators, are known to be ubiquitinated and degraded by the 26S proteasome, which consists of the 20S proteolytic core capped by the 19S regulatory complex (36). Specifically, the transcriptional activity of RARs is linked to proteasome-mediated pathways by two types of kinases, CDK7 and p38MAPK. Double phosphorylation of RARA, first in the ligand binding domain by PKA and then in the AF-1 domain by CDK7 kinase associated with cyclin H within TFIIH, a part of general transcriptional complex, is required for *at*RA-induced ubiquitination of the receptor and degradation by proteasomes (36, 41). This process of TFIIH-mediated phosphorylation of RARs, which marks RARs for degradation, implies that the proteasome mediated protein degradation is required for the transcriptional activation of RARs.

p38MAPK also phosphorylates the N-terminal domain of RARG and marks the receptor for ubiquitination and proteasome degradation, perhaps by controlling the recruitment of ubiquitin ligases (42). In addition, proteasome without proteolysis initiated by p38MAPK seems to be important for RAR-dependent transcription. For example, phosphorylation by p38MAPK in AF-1 domain and the recruitment of SUG-1, one of the six ATPase of the 19S regulatory complex of the 26S proteasome, to the AF-2 domain are required for RA-induced RARG transactivation (43). Recently, SUG-1 has been shown to interact with SRC-3, a coactivator of RARA, to mediate the transcription of RA target genes (44). Meanwhile, the ratio between SUG-1 and SRC-3 is crucial for the control of RARA functioning: when present in excess amounts, SUG-1 blocks the activation of RARA target genes and the degradation of RARA that occurs in response to RA, via its ability to interfere with the recruitment of SRC-3 and other coregulators at the AF-2 domain of RARA (44).

SUMOYLATION

Sumoylation entails a covalent attachment of small ubiquitin-like modifier (SUMO), ligated by a isopeptide bond to a lysine residue within the tetrapeptide motif, Ψ-Lys-X-Glu (where Ψ is a large hydrophobic amino, X is any amino acid), of the protein targets (45). There are four known members SUMO-1/Smt3C, SUMO-2/Smt3A, SUMO-3/Smt3C and SUMO-4/Smt4H. SUMO-2 and -3 share 95% amino-acid sequence identity, and each shares about 47% sequence identity with SUMO-1. Similar to ubiquitin, SUMO-2 and -3 have their own sumoylation sites, and thus, they can form a polysumoylated chain, however, the functional significance, if any, of SUMO polychains *in vivo* has not been established (45). On the other hand, SUMO-1 cannot conjugate to itself. As for SUMO-4, which has a restricted pattern of expression, with the highest level reported in the kidney (46), it may function exclusively as a noncovalent interacting protein (47). Sumoylation, similar to ubiquitination, occurs through a three-step enzymatic process, which includes SUMO activation by the E1 enzyme, SAE1/SAE2, SUMO conjugation to the target substrate by the E2 enzyme, UBC9, and SUMO ligation by E3-like ligases, which are thought to be substrate specific (45). In reverse, a family of SUMO/sentrin specific peptidases

(SENPs) functions in proteolytic processing of SUMO precursors and in deconjugation of SUMO from proteins (48).

Recently, sumoylation has surfaced as an important mechanism in modulating cell-cycle progression, regulation of transcription, and sex chromosome inactivation (45). The functional consequences of sumoylation are quite diverse and appear to be substrate-specific. Sumoylation has been reported to regulate transcription, nuclear-cytoplasmic transport, protein stability, and protein-protein interactions (45). For example, the transcriptional corepressors CtBP is normally nuclear, but mutation of its single SUMO modification site resulted in a cytoplasmic localization and a failure to repress transcription(45). The transcriptional activity of peroxisome proliferator-activated receptor gamma is modulated by SUMO-1 (49). Negative modulation of RXRA transcriptional activity by SUMO-1 modification can be reversed by SUMO-specific protease SUSP1 (50). Additionally, mutations of sumoylation sites of the mineralocorticoid receptor (MR) enhances MR activity on a minimal promoter, but not on the MMTV promoter, indicating a promoter-selective effect (51).

MOLECULAR CHAPERONES

Molecular chaperones such as heat shock protein 90 (Hsp90) machinery have been highlighted to be vital in modulating the functions of nuclear receptors (52). Nuclear receptor association with chaperones occurs in an ordered, step-wise fashion, and is necessary for the maintenance of unliganded receptor in a state ready to bind and respond to hormone (53). Chaperones modulate how nuclear receptors respond to hormone and activate target genes, such as their hormone binding properties, posttranslational modification, subcellular localization, interactions with chromatin and transcription cofactors, as well as proteolytic half-life (53). For instance, glucocorticoid receptor (GR) resides predominantly in the cytoplasm as a multiprotein heterocomplex that contains heat shock protein 90 (Hsp90), Hsp70, and an immunophilin, such as FKBP51, FKBP52 or Cyp-40 (52, 54, 55). The activation of GR appears to involve the chaperone Hsp90 (56, 57). Hsp90 is a chaperone for maintaining an appropriate ligand-binding conformation for steroid receptors, but it also participates in the nuclear–cytoplasmic shuttling of

steroid receptors (58, 59). The GR-Hsp90 complex is in a dynamic state of assembly and disassembly (55); however, association with Hsp90 is necessary for the receptor movement (59, 60). It has been proposed that GR moves through the cytoplasm to the nucleus in the complex form with Hsp90 and the immunophilin acting as a protein transport unit of the transportsome (61, 62). The dynamic heterocomplex of GR-Hsp90-FKBP52 associates with tubulin and the microtubule motor protein dynein, which shuttles the complex along microtubular tracks toward the nucleus which ultimately release GR into the nucleus (63, 64). Once it enters the nucleus, Hsp90 dissociates from GR and releases GR in the nucleus for it to mediate the transcription of target genes (65). Moreover, the ligand binding and transcriptional activation of RAR was impaired in a yeast lacking Hsp90 (66). Additionally, the Hsp90 prolongs receptor proteolytic half-life because pharmacological inhibition of Hsp90, which short circuits the assembly cycle, promotes rapid receptor degradation in cells (67).

Recently, molecular chaperones in the endoplasmic reticulum (ER) are attracting attentions. Approximately, one-third of all proteins in eukaryotes are targeted to the secretory pathway, and the first compartment encountered by these diverse proteins is the ER (68). The ER is the site of synthesis and maturation of proteins destined for secretion, for the plasma membrane, and for the secretory and endocytic organelles. Misfolded and incompletely assembled proteins are common side products of protein synthesis into the ER, and they are retained in the ER until folded correctly (69, 70) or eventually exit to the cytoplasm and degraded by ERassociated degradation machinery (ERAD) (71). The ER contains molecular chaperones such as BiP, calnexin, calreticulin, GRP94, protein disulfide isomerase (PDI), ERp57 and ERp72. These factors are not only responsible for assisting in the folding and assembly process, but also serve as retention anchors for immature proteins (72). Calreticulin and calnexin are two homologous ER-resident lectins, and bind to almost all soluble and membrane-bound glycoproteins synthesized in this compartment (73). They specifically associate with glycoproteins that have monoglucosylated trimming intermediates of the N-linked core glycans (74). Together with ERp57, a thiol oxidoreductase, with which they form complexes, they mediate the retention and promote proper folding of glycoprotein substrates. If the substrate has attained its native conformation, it is sent to Golgi.

If not, the substrate is either subjected to additional folding cycles or is selected for ERAD. ERAD involves retrotranslocation of misfolded proteins to the cytoplasm, where they are deglycosyled, ubiquitinated by ER-bound ubiquitin-conjugating enzymes such as the Ubc6e E2 enzyme and E3 RMA1 ubiquitin ligase, and degraded by proteasomes (71, 75). For instance, upon proteasome inhibitor treatment in mammalian cells, a misfolded variant of α_1-antitrypsin with its glycans in the $Glc_1Man_8GlcNAc_2$ form is retained in the ER and stably associated with calnexin (76). On the other hand, the ER environment is more oxidizing than the cytoplasm, and this favors the formation of disulfide bonds. This process is catalyzed by protein disulfide isomerases (PDIs) such as ERp57, which possess thiol oxidoreductase activity that further aids proteins to reach their native conformation. Besides enabling the *de novo* formation of disulfide linkage, PDIs also isomerize non-native disulfide bonds, thus facilitating the acquisition of the native state. One recently characterized human PDI homologue, ERDJ5, contains four canonical thioredoxin-like active-site CXXC motifs and is a BiP cofactors (77).

Of interest, GRp58, identical to ERp57, ERp60/ERp61, and PDI-Q2, is also named $1,25D_3$-MARRS (membrane-associated, rapid response steroid binding) receptor. $1,25D_3$-MARRS receptor is the receptor of vitamin D metabolites, $1,25(OH)_2D_3$, that helps regulate the calcium and phosphate metabolism of animals for immediate to long-term homeostasis. Upon $1,25(OH)_2D_3$ binding, $1,25D_3$-MARRS receptor translocates to the nucleus, where it may interact with transcription factors and/or DNA (78, 79). Moreover, the receptor activates both PKC and PKA which may modulate the activity of transcription factors (80).

SUMMARY

Spermatogenesis is a complex process that takes place in testes (1). Vitamin A is essential for spermatogenesis and one of the retinoid receptors, RARA, is critical for spermatogenesis (81) (29). In testes, RARA may play a major role in the maintenance of the stages of the spermatogenic cycle (31, 32). In Sertoli cells it may promote the survival and development of early meiotic prophase spermatocytes,

whereas in germ cells it functions to increase the proliferation and differentiation of spermatogonia before meiotic prophase (30). Being a transcription factor, RARA mediates the transcription of downstream genes, but RARA itself can be regulated by post-translational modifications and cofactor bindings (21, 82). Hence, to understand how RARA is regulated is important to uncover the role of RARA in testes.

In Chapter 2, a yeast two-hybrid screening was conducted to identify RARA-interacting proteins with RARA as the bait and proteins expressed from 20-day-old rat Sertoli cells cDNA library as the prey. 15 candidate genes were identified and, among the 15 candidates, herein, small ubiquitin like modifier-2 (SUMO-2) (Chapter 3 and 4) and glucose related-receptor 58 (GRp58) (Chapter 5), which showed the highest interaction scores, were investigated further.

In Chapter 3, the regulation of RARA stability, nuclear localization and transcriptional activity by SUMO-2 modification of RARA was examined. We demonstrate that the SUMO-2 modification of RARA is a novel posttranslational modification of RARA. SUMO-2 modification of RARA regulates the stability of RARA and RARA-SUMO conjugates dependent on the ligand and the amount of SUMO-2. Sumoylation of RARA at specific sumoylation sites influences its nuclear localization and transcriptional activity, either positively or negatively, in a site-dependent manner. These data indicate that manipulation of SUMO-2 could be an alternative strategy to regulate the functions of RARA.

In Chapter 4, the cellular localization and protein expression of SUMO-2/3 in testes were examined. The amino-acid sequences of SUMO-2 and SUMO-3 are 95% similar to each other. We found that the protein expression of SUMO-2/3 was regulated by RA, but not through RARA signaling pathway, whereas the cellular localization of SUMO-2/3 was modulated by RA through RARA signaling pathway. These studies suggest that the relationship of RA, RARA and SUMO-2 is finely tuned in testes.

In Chapter 5, the regulation of RARA nuclear localization by GRp58 was investigated. We demonstrate that GRp58 is a novel molecular chaperone required for nuclear trafficking of RARA. The *at*RA ligand is able to mobilize both RARA and GRp58 into the nucleus, where GRp58 targets RARA to the endoplasmic reticulum (ER), before it dissociates from RARA. Additionally, studies with sulfhydryl

modifying agents allow us to speculate that the thiol-oxidoreductase activity of GRp58 on cysteine residues in RARA might be important for the ligand binding to RARA and subsequent nuclear trafficking of RARA. These results suggest that manipulation of GRp58 could be an alternative strategy to modulate the functions of RARA.

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CHAPTER 2

IDENTIFICATION OF PHYSICALLY INTERACTING PARTNERS OF RETINOIC ACID RECEPTOR ALPHA IN SERTOLI CELLS

This chapter was written as a summary for the yeast two hybrid screening.

IDENTIFICATION OF PHYSICALLY INTERACTING PARTNERS OF RETINOIC ACID RECEPTOR ALPHA IN SERTOLI CELLS

2.1 INTRODUCTION

Genetic studies have shown that RARA is critical for spermatogenesis, as shown by the sterility phenotype of male mice lacking *Rara* gene (1). Further characterization revealed a role of RARA in the synchronization of the spermatogenic cycle (2, 3). A normal proportion of the spermatogenic stages of the seminiferous epithelial cycle could not be maintained without RARA; instead, a major disorganization of germ-cell association was observed (2, 3). In addition, our lab has demonstrated (4) that RARA may function in Sertoli cells to promote the survival and development of early meiotic prophase spermatocytes, whereas RARA in germ cells may function to increase the proliferation and differentiation of spermatogonia before meiotic prophase. Hence, it is clear that RARA is an essential component for normal spermatogenesis in the testis.

To identify RARA interacting protein in Sertoli cells, a yeast two-hybrid screening was performed with RARA as the bait and proteins expressed from a Sertoli cell cDNA library as the prey. Yeast two-hybrid screening, pioneered by Fields and Song in 1989 (5), was originally designed to detect protein-protein interaction using GAL4 transcriptional activator of the yeast. Since then, the yeast two-hybrid screening has become a practical technique used to discover protein-protein and protein-DNA interactions by testing for physical interactions (such as binding) between two proteins or a single protein and a DNA molecule, respectively. The principle of this assay is the binding of a transcription factor to an upstream activating sequence (UAS) activates the transcription of downstream reporter gene. Here the transcription factor is split into two separate fragments, named the binding domain (BD) and the activation domain (AD), respectively. This screening often utilizes a genetically engineered yeast strain, lacking the ability to synthesize certain nutrients (usually amino acids), required for the survival in the media without these nutrients. In this screening, a plasmid encoding the protein fused to BD referred as the bait and a plasmid encoding the protein fused to AD referred as the prey are simultaneously introduced into the yeast strain

plated in media lacking certain nutrients after transformation. If the bait protein interacts with the prey protein, the interaction would lead to the indirect connection between the BD and AD, resulting in the AD in proximity to the UAS, activating the transcription of downstream report genes. Thus, a successful interaction between the bait and the prey is directly linked to a phenotypical change.

2.2 EXPERIMENTAL PROCEDURES

Plasmid Constructs – A hRARA cDNA from LRARaSN (6) provided by Dr. Steve Collins (Fred Hutchinson Cancer Research Center, Seattle, WA) was subcloned into pBD-GAL4 vector (Stratagene, La Jolla, CA) to generate pBD-h*RARA* expression construct. Additionally, rRXRA cDNA from rat pRxra provided by Dr. Jan-Ake Gustafsson, (Karolinska Institutet, Huddinge, Sweden) was subcloned into pAD-GAL4 vector (Stratagene, La Jolla, CA) to generate pAD-r*Rxra* expression construct.

Antibodies – Rabbit anti-RARA and rabbit anti-RXRA antibodies were obtained from Santa Cruz biotechnology, Inc. (Santa Cruz, CA).

Yeast Two-Hybrid Screening – The Matchmaker Two-Hybrid System 3 (Clontech Laboratories, Inc., Mountain View, CA) was utilized to screen a cDNA expression library from 20-day-old rat Sertoli cells (7), with RARA as the bait. One of the yeast strain AH109 was pretransformed with pBD-h*RARA* expression construct. The other yeast strain Y187 was pretransformed with the rat Sertoli cell cDNA expression library in pAD-GAL4. These two pretransformed yeast strains were mated together in a 2L flask, and incubated at 30^{o}C overnight with gentle shaking (45 rpm). After 24 hrs mating, the transformants were plated on a synthetic dropout medium (SD) with various stringencies: SD/-Trp, SD/-Trp/-His, SD/-Trp/-Ade or SD/-Ade/-His/-Leu/-Trp, respectively lowest to highest, at 30^{o}C for 4 days. Positive colonies that grew in the highest stringency medium (SD/-Ade/-His/-Leu/-Trp) were re-streaked and measured for β-galactosidase activity (LacZ^{+} colonies) using the filter-lift assay according to the procedure described in Yeast Protocols Handbook (Clontech Laboratories, Inc.). Positives colonies

exhibiting high β-galactosidase activity were further characterized. Plasmid DNA from positive colonies was isolated, propagated in *E.coli* DH5α (Invitrogen), and sequenced to identify clones.

Cell culture and RNA extraction – Primary Sertoli cells were isolated from the testes of 20-day-old rats by sequential enzymatic digestions (8). Decapsulated testis fragments were digested first with 0.25% (w/v) trypsin (Gibco BRL, Gaithersburg, MD) to remove the interstitial cells, and then with collagenase (0.7 mg/mL, Sigma-Aldrich), and maintained in Ham's F-12 medium (Invitrogen Laboratories, Carlsbad, CA) in a 32 ^{o}C 5% CO_2 atmosphere. Animal protocols, following the NIH guidelines, were approved by the Institutional Animal Care and Use Committee of Washington State University.

Total RNA, isolated from different tissues of 20-day old SD rats using TRIzol reagent, was used to synthesize cDNA using iScript cDNA Synthesis Kit (Bio-Rad). The generated cDNAs were substrates to conduct PCR with primers specific for rat SUMO-2, SUG1, GRP58, DMRT1 and Shbg.

2.3 RESULTS AND DISCUSSION

To identify physically interacting protein(s) of RARA, a yeast two-hybrid screening was conducted with RARA as the bait and proteins expressed from a rat Sertoli cell cDNA library, constructed using Sertoli cell mRNA from 20-day-old rats, as the prey. The Sertoli cell cDNAs, fused to GAL4 transactivation domain [GAL(AD)-cDNA], were pre-transformed into the Y187 yeast strain, engineered with a LacZ reporter gene, controlled by GAL1UAS (UAS_{GAL4}). Meanwhile, another engineered yeast strain AH109, with three reporter selection markers, a histidine auxotrophic marker (HIS3), an adenosine-2 (ADE2) reporter gene, and a LacZ reporter gene, was pre-transformed with a plasmid, encoding a chimeric protein comprising GAL4DNA-binding domain (DBD) and full-length hRARA. Expression of the RARA bait in the yeast strain AH109 was confirmed by Western blot analysis with anti-RARA antibody (Fig. 2.1, A).

In the yeast two-hybrid screening, one major cause of false-positive results is called autonomous activating activity (in which the bait fusion protein contains an activating sequence so that both binding

and activating domains are expressed by a single protein). To exclude this possibility, AH109 yeast cells were transformed with pBD-h*RARA* or empty pBD vector. After 4 days of incubation at 30 ^{o}C, colonies grew on SD/-Trp/X-α-gal plates, but no colonies were observed on SD/-Trp/-His/X-α-gal and SD/-Trp/-Ade/X-α-gal plates, indicating that the bait alone could not activate transcription. In addition, a two-hybrid interaction between RARA and RXRA, a known heterodimeric partner of RARA, encoded by a pAD-*Rxra* plasmid, was performed. In the screening, pAD-*Rxra* and pBD-h*RARA* were cotransfromed into AH109 and the transformants were plated on various media as mentioned previously. After 4 days of incubation, colonies were grown on SD/-Ade/-His/-Leu/-Trp, suggesting that the yeast two-hybrid screening was working properly. Expression of the RXRA in the yeast strain Y187 was confirmed by Western blot analysis with anti-RXRA antibody (Fig. 2.1, B).

To identify the physically interacting protein(s) of RARA, these two pre-transformed AH109 and Y187 yeast strains were mated together for overnight. After mating, the mating mixtures were plated on SD/-Ade/-His/-Trp medium and incubated at 30 ^{o}C for 4 days. Approximately, $1.1x10^{7}$ HIS and ADE2 positive colonies were obtained in this primary screening. When colonies grew up to around 1 mm in diameter, a β-gal colony-lift filter assay was performed and eliminated most of false positive colonies. 69 HIS, ADE2 and LacZ positive colonies were obtained. To further eliminate duplicates in 69 remaining colonies, plasmid DNA was extracted from the remaining colonies and transformed into *E.coli* DH5α (Invitrogen). Plasmid DNA was extracted from DH5α and digested with a frequent-cutter restriction enzyme, *Alu*I and analyzed on DNA agarose gels. Meanwhile, the plasmid DNA was also sequenced using the primer, 5'- GGTTGGACGGACCAAACTGCGTATA and a T7 primer. By comparing the digested DNA fragment pattern and DNA sequences, we were able to decrease the number from 69 to 24 as listed (Table 2.1).

To further verify the interaction between RARA and these clones, AH109 were transformed with pBD-h*RARA* and plasmid DNA isolated from the remaining 24 clones, and plated on SD/-Ade/-His/-Trp medium and incubated at 30 ^{o}C for 4 days. When colonies grew up to around 1 mm in diameter, a β-gal colony-lift filter assay was performed. Only 15 positive clones were obtained as shown in Table 2.2,

which were arranged by their β-gal activities from high (+++), medium (++) to low (+). The higher β-gal activity means the stronger interaction.

Among the top 5 candidates, SUMO-2 (small ubiquitin related modifier-2), which has the highest β-gal activity, belongs to the SUMO family, known to modify target proteins by attaching one or multiple SUMOs to the target protein, affecting its transcription, nuclear-cytoplasmic transport, protein stability, and protein-protein interactions (9). SUG1, one of the six ATPase of the 19S regulatory complex of the 26S proteasome, has been shown to interact with RARA (10) and, interestingly, phosphorylation by p38MAPK in AF-1 domain and the recruitment of SUG-1 to the AF-2 domain are required for RA-induced RARG degradation and transactivation (11). GRp58, glucose-regulated protein 58, is a glycoprotein-specific thiol-oxidoreductase and an isoform of protein disulfide isomerase (PDI), which catalyzes the process of thiol oxidoreductase activity as well as isomerizes non-native disulfide bonds, thus facilitating the acquisition of the native state for a substrate protein (12). Additionally, one of the candidates were DMRT1, (double sex and mab-3 related transcription factor 1), a key regulator of male development in flies and nematodes (13). The last one was Shbg, sex hormone binding globulin precursor, a glycoprotein that binds to sex hormones, specifically testosterone and estradiol (14).

Since these five candidates have the highest β-gal activities, they were chosen to be further characterized. To determine if they are expressed in Sertoli cells, total RNA was isolated from primary Sertoli cells and used as the template for PCR with forward primers containing NotI and reverse primers containing ClaI, specific to the full-length of rat SUMO-2, SUG1, GRp58, DMRT1 and Shbg, respectively. RT-PCR showed that SUMO-2, SUG1, GRp58, DMRT1 and Shbg were present in primary Sertoli cells (Fig. 2.2). To characterize the functions of these candidates in mammalian cells, the PCR fragment generated previously was subcloned into pFLAG-CMV-2 vector (Sigma-Aldrich). Although SUG1 couldn't be subcloned into pFLAG-CMV-2 vector, the rest were successfully subcloned into pFLAG-CMV-2 vector. Two of these constructs, pFLAG-*Sumo-2* and pFLAG-*GRp58*, have been verified by Western blot analyses and were used in the studies described in Chapter 3-5. Additionally, Sumo-2 was also subcloned into pQE-Trisystem vector (Qiagen Inc., Valencia, CA) to generate pHis-

Sumo-2 expression construct used in Chapter 3. Together, we obtained 15 potential RARA interacting proteins and two of them, SUMO-2 and GRp58, were chosen to be further investigated in the following chapters.

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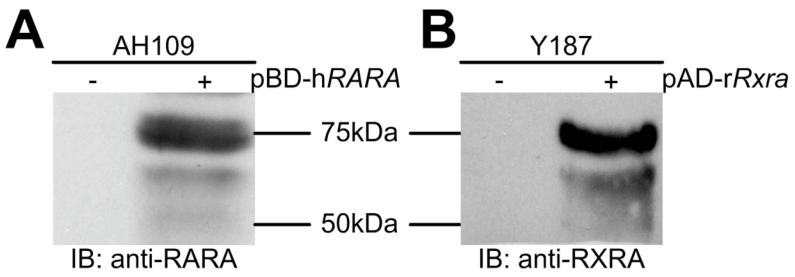

Fig. 2.1 **Expression of pBD-hRARA and pAD-rRXRA was verified by Western blot analyses.** A-B), The yeast strain AH109, transfected with pBD empty vector (-) or pBD- hRARA (+) (A), and Y187, transfected with pAD empty vector (-) or pAD-rRxra (+) expression constructs, were lysed after 24 h and analyzed by immunoblotting (IB) with anti-RARA (A) or anti-RXRA (B) antibodies.

	Colony#	Symbol	Pubmed Access #	Name	Similar Colonies
1	1	unknown	unknown	unknown	1,2
2	3	Sc5d	NM_053642	Sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog (S. cerevisae)	3,4,66,67
3	5	Vdp	NM_019379	Rattus norvegicus general vesicular transport factor p115 mRNA	5,6,7
4	8	Col4a3	NM_007734	Mus musculus procollagen, type IV, alpha 3	22
5	10	Rps6	NM_017160	Rattus norvegicus ribosomal protein S6	10,11
6	14	Phf12	NM_001013117	Rattus norvegicus PHD finger protein 12	12,28,60
7	18	Itgb1bp1	NM_008403	Mus musculus integrin beta 1 binding protein 1	18,21,25,61,69
8	19	Prosaposin	BC061759	Rattus norvegicus prosaposin; Rat sulfated glycoprotein 1	19,63
9	20	Cdc42se2	NM_178626	Mus musculus CDC42 small effector 2	13
10	24	Lamr1	NM_017138	Rattus norvegicus laminin receptor 1 (67kD, ribosomal protein SA) (),	23,24
11	26	P2rY2	NM_017255	Rattus norvegicus purinergic receptor P2Y, G-protein coupled 2	26,27,56
12	31	Azi2 predicted	XM_236698	Rattus norvegicus 5-azacytidine induced gene 2(prediceted)	
13	32	Cipp	BC019701	Mus musculus InaD-like (Drosophila),	
14	33	Txndc4	NM_029572	Mus musculus thioredoxin domain containing 4 (endoplasmic reticulum)	30
15	34	Shbg	NM_012650	Sex hormone-binding globulin precursor	35,36
16	37	Map1lc3b	NM_022867	Rattus norvegicus microtubule-associated proteins 1A/1B light chain 3	37,38,42
17	39	DMRT-1	NM_053706	Rattus norvegicus doublesex and mab-3 related transcription factor 1	45
18	40	GRp58	NM-017319	Rattus norvegicus glucose regulated protein, 58 kDa	40,41
19	43	Txndc9	BC060541	Rattus norvegicus thioredoxin domain containing 9	
20	44	non-muscle myosin alkali light chain	S77858	non-muscle myosin alkali light chain	
21	46	Sumo2	NM_133594	SMT3 suppressor of mif two 3 homolog 2 (yeast)	9,15,16,17,47,48
22	50	SUG1	NM_031149	Rattus norvegicus for proteasomal ATPase (LOC81827)	49,50,51,52,53,58,59
23	54	Atbf1 (predicted)	BC060729	Mus musculus AT motif binding factor 1, mRNA	54,55,57,62,64
24	68	NADH dehydrogenase subunit 1	AJ428514	Rattus norvegicus complete mitochondrial genome, wild-caught animal	65

TABLE 2.1. **Potential RARA interacting proteins obtained from the first round yeast two-hybrid screening.**

	Symbol	Pubmed Access #	Name	β–gal activity
1	Sumo2	NM_133594	SMT3 suppressor of mif two 3 homolog 2 (yeast)	***
2	SUG1	NM_031149	Rattus norvegicus for proteasomal ATPase	***
3	GRp58	NM-012319	Rattus norvegicus glucose regulated protein, 58 kDa	***
4	DMRT1	NM_053706	Rattus norvegicus doublesex and mab-3 related transcription factor 1	**
5	Shbg	NM_012650	Sex hormone-binding globulin precursor	**
6	Sc5d	NM_053642	Sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog (S. cerevisae)	**
7	Vdp	NM_019379	vesicle docking protein	**
8	Map11c3b	NM_022867	Rattus norvegicus microtubule-associated protein 1 light chain 3	**
9	CDC42se2	NM_178626	Mus musculus CDC42 small effecter 2	**
10	P2ry2	NM_017255	Rattus norvegicus purinergic receptor P2Y, G-protein coupled 2	**
11	Non-muscle myosin alkali light chain	S77858	Non-muscle myosin alkali light chain	**
12	Rps6	NM_017160	Rattus norvegicus ribosomal protein S6	*
13	Phf12	NM_001013117	PHD finger protein 12	*
14	Atbf1 (predicted)	BC060729	Mus musculus AT motif binding factor 1	*
15	Unknown	Unknown	Unknown	*

TABLE 2.2. **Potential RARA interacting proteins obtained from the final round yeast two-hybrid screening.** The 15 candidates were arranged according to their β-gal activity. ***, the highest activity; **, the medium activity; *, the lowest activity.

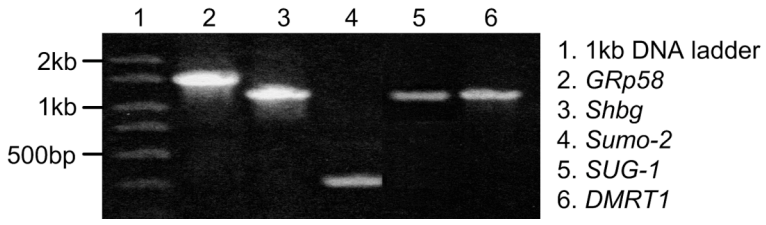

FIGURE. 2.2 **SUMO-2, GRp58, Shbg, SUG-1, DMRT1 mRNA were expressed in primary Sertoli cells.** Total RNA, isolated from 20-day-old rat Sertoli cells, was utilized to synthesize cDNA used as the temple for PCR with specific primers for each gene.

CHAPTER 3

SUMO-2 CONJUGATION OF RETINOIC ACID RECEPTOR ALPHA MODULATES ITS STABILITY, SUBCELLULAR LOCALIZATION AND TRANSCRIPTIONAL ACTIVITY

This manuscript was submitted to Molecular Endocrinology. I am the primary author and the Fig. 3.3B-C was completed by Nadine C. Santos.

SUMO-2 Conjugation of Retinoic Acid Receptor Alpha Modulates its Stability, Subcellular Localization and Transcriptional Activity

Abbreviated title: SUMO-2 Modification of RARA

Li Zhu, Nadine C. Santos, Kwan Hee Kim

School of Molecular Biosciences and Center for Reproductive Biology, Washington State University, Pullman, Washington 99164

Address correspondence to: Kwan Hee Kim, School of Molecular Biosciences, Washington State University, Pullman, WA 99164-4234, Tel: 509-335-7022, Fax: 509-335-1907, Email: khkim@wsu.edu

Reprint request to: Kwan Hee Kim, School of Molecular Biosciences, Washington State University, Pullman, WA 99164-4234

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3.1 Abstract

Post-translational modifications of retinoic acid receptor alpha (RARA) have emerged as an important mechanism to control its transcriptional activity. Here, we demonstrate that RARA is covalently modified by SUMO-2 and this affects the protein stability, subcellular localization, and transcriptional activity of RARA. Lys 399 (K399) of RARA is the only site available for sumoylation in the absence of all-*trans* retinoic acid (*at*RA), whereas two additional sites, K166 and K171, are revealed in the presence of *at*RA. Interestingly, *at*RA increased RARA sumoylation, while SUMO-2 had a biphasic influence on RARA and sumoylated RARA levels. They were both high at the endogenous level of SUMO-2, declined at intermediate levels of SUMO-2, and then increased at high levels of SUMO-2. Mutation of K399 to Arg (K399R) shifted RARA from the cytoplasm to the nucleus in the absence of *at*RA, and even more interestingly, this mutant was found primarily in the cytoplasm in the presence of *at*RA, indicating a complete loss of the *at*RA-controlled nuclear trafficking. Consistently, the K399R mutation of RARA abolished its transcriptional activity. In contrast, the triple RARA mutant has lost the *at*RA control of subcellular localization, but gained enhanced transcriptional activity, indicating that sumoylation on K166 and K171 is inhibitory to the RARA-mediated transcription. SUMO-2 on K166 and K171 could be removed by SUMO/sentrin-specific peptidase 3 and 6, which are highly expressed in Sertoli cells and exclusive for a SUMO-2/3 polychain. Manipulation of SUMO-2 conjugation and de-conjugation can control the function of RARA, which is essential in the testis.

3.2 Introduction

Retinoic acid receptor alpha (RARA) belongs to the superfamily of steroid/thyroid hormone receptors with highly conserved domains. Each receptor has an N-terminal A/B region that harbors the ligand-independent activation function-1 (AF-1), a central C region containing DNA-binding domain (DBD), and a C-terminal E region that encompasses the ligand-binding domain (LBD) with a dimerization interface and a ligand-dependent transactivation domain, AF-2 (1, 2). Binding of a ligand to the receptor induces a major conformational change to the receptor (3), creating a new AD core interface on the AF-2, for the recruitment of chromatin remodeling cofactors (1, 2). The liganded receptor with its retinoid X receptor (RXR) heterodimer partner then binds the retinoic acid response element (RARE) on target genes and modulates their transcription in the nucleus. The natural ligand of the retinoic acid receptor family, RARA, RARB, and RARG, is all-*trans* retinoic acid (*at*RA) (4).

One of the *Rar* genes, the *Rara* gene is critical for spermatogenesis, as shown by a sterility phenotype in the null mutant males (5). Similar to testes from animals on a vitamin A-deficient diet (6), testes of *Rara*-null mutants displayed increased apoptosis in early meiotic spermatocytes (7). Further characterization of the testes from *Rara*-null mice revealed a role of RARA in the maintenance of the stages of the spermatogenic cycle (8, 9). Dissecting the cell-type functions by spermatogonial transplantation experiments, we found that RARA may function in Sertoli cells to promote the survival and development of early meiotic prophase spermatocytes, whereas RARA in germ cells functions to increase the proliferation and differentiation of spermatogonia before meiotic prophase (7). Hence, it is clear that understanding how the transcriptional activity of RARA is regulated is central to unraveling the role of RARA in Sertoli and germ cells.

Post-translational modifications of RARA have been shown to play a major role in the regulation of its subcellular localization, degradation, and transcriptional activity (10-14). Follicle stimulating hormone (FSH), via cAMP and protein kinase A (PKA), inhibited *at*RA-induced RARA nuclear localization, reduced the steady state level of RARA, and decreased the RARE-dependent transcription in a mouse Sertoli cell line (MSC-1) stably transfected with the FSH receptor cDNA (10). Another study showed that

RARA can be positively regulated by protein kinase C and mitogen-activated protein kinase in Sertoli cells, independent of the ligand (11). Moreover, double phosphorylation of RARA, first in the ligand binding domain by PKA and then in the AF-1 domain by CDK7 kinase, was required for *at*RA-induced ubiquitinylation of the receptor and degradation by proteasomes (12, 13).

To better understand the molecular elements regulating the transcriptional activity of RARA in Sertoli cells, we performed yeast two-hybrid screening using RARA as the bait and the protein products of a primary Sertoli cell cDNA expression library as the prey to identify proteins that interact with RARA in Sertoli cells. One of the proteins that interacted strongly with RARA was SUMO-2, involved in sumoylation, which has surfaced as an important mechanism in modulating cell-cycle progression, regulation of transcription, and sex chromosome inactivation (15). Sumoylation entails a covalent attachment of small ubiquitin-like modifier (SUMO), ligated by a isopeptide bond to a lysine residue within the tetrapeptide motif, Ψ-Lys-X-Glu (where Ψ is a large hydrophobic amino, X is any amino acid), of the protein targets (15).

There are four known members in the SUMO family: SUMO-1/Smt3C, SUMO-2/Smt3A, SUMO-3/Smt3C and SUMO-4/Smt4H. SUMO-2 and -3 share 95% amino-acid sequence identity, and each shares about 47% sequence identity with SUMO-1. Similar to ubiquitin, SUMO-2 and -3 have their own sumoylation sites, and thus, they can form a poly SUMO-2/3 chain (15), whereas, SUMO-1 cannot conjugate to itself. Although the functional significance of SUMO-2/3 polychains at a molecular level has not been established, it is clear that a single SUMO-1 is less bulky than a polychain of SUMO-2/3. Moreover, it cannot be ignored that the cells are expending much energy to express SUMO/sentrin-specific peptidases (SENPs), which can discriminate between SUMO-2/3 on a polychain and the first SUMO or one SUMO-1 directly on the substrate protein (16). As for SUMO-4, which has a restricted pattern of expression, with the highest level reported in the kidney (17), it may function exclusively as a noncovalent interacting protein (18). Sumoylation, similar to ubiquitinylation, occurs through a three-step enzymatic process, which includes SUMO activation by the E1 enzyme, AOS1/UBA2 (SAE1/SAE2), SUMO conjugation to the target substrate by the E2 enzyme, UBE2I (UBC9), and SUMO ligation by E3-

like ligases, which are thought to be substrate specific (15). In reverse, a family of SENPs functions in proteolytic processing of SUMO precursors, in editing and deconjugation of SUMO from the target protein (16).

The functional consequences of sumoylation are substrate-specific and, reflecting this, quite diverse. Sumoylation has been reported to regulate cell cycle progression, transcription, nuclear-cytoplasmic transport, protein stability, and protein-protein interactions (15). In the testis, SUMO-1, SUMO-2/3, and UBC9 are demonstrated to be expressed in male germ cells during meiosis and in spermiogenesis (19) and SUMO-1 in Leydig and Sertoli cells (20). It has been postulated that SUMO-1 is involved in heterochromatin organization, meiotic centromere function, and nuclear reshaping (20) and SUMO-2/3 in the metaphase I function of spermatocytes (19). In Sertoli cells, there appears to be an inverse correlation between androgen receptor function and SUMO-1 expression (21), but the molecular role of SUMO-2/3, which can form a poly SUMO-2/3 chain, is poorly understood in Sertoli cells. Since human patients with SUMO-2/3 hypo-sumoylation or hyper-sumoylation were found to have azoospermia, hypospermatogenesis or reduction in spermatogenesis (22), it is important to investigate SUMO-2/3 substrates in the testis. Here, we demonstrate that RARA is modified by SUMO-2 in Sertoli cells and COS-7 cells, and this influenced the protein stability, subcellular localization, and transcriptional activity of RARA.

3.4 Results

RARA has SUMO acceptor sites

SUMO-2 expressed from a Sertoli cell cDNA library was identified in our yeast two-hybrid screen as interacting with RARA. *Sumo-2* cDNA isolated from the LacZ-positive yeast clone was sequenced. The translated protein sequence of *Sumo-2* cDNA was 100% identical to SUMO-2 protein sequences derived from mouse (NM_133354), rat (NM_133594), and human (NM_006973) sequence in the GenBank database (Fig. 3.1A) and contained VKTE, which matched the tetrapeptide sumoylation consensus motif, ΨK*x*D/E (Fig. 3.1A). SUMO-2/3 is known to covalently attach to the lysine reside within the tetrapeptide

sumoylation motif of itself, making a poly-SUMO-2/3 chain (15). To identify SUMO acceptor sites on RARA, SUMOplot™ (http://www.abgent.com/doc/sumoplot) was used. In RARA, we identified four putative SUMO acceptor sites (Fig. 3.1B): K399 located in the ligand-binding domain (LBD) with the highest score (the highest possibility to accept SUMO); K171 and K166, in the nuclear localization signal (NLS), with intermediate scores; and K147, in the DNA-binding domain (DBD), with the lowest score. All sites are highly conserved in different species (Fig. 3.1C).

RARA can be covalently modified by SUMO-2

To determine whether RARA can be conjugated by SUMO-2, COS-7 cells, which have little or no endogenous RARA (Fig. 3.10 A), were transfected with pFLAG-*RARA* and pFLAG-*Sumo-2* cDNA constructs, immunoprecipitated with either anti-RARA or anti-SUMO-2/3 antibody, and then immunoblotted with either anti-SUMO-2/3 (Fig. 3.2A) or anti-RARA antibody (Fig. 3.2B). Western blot analyses yielded intense complexes around 150kDa and a minor band around 70kDa, recognized by both anti-SUMO-2/3 and anti-RARA antibodies. Since the molecular weight of SUMO is ~15kDa by SDS-PAGE, a covalent attachment of SUMO to a target protein could induce a mobility shift, the amount of the upward shift dependent on the number of SUMOs attached to the protein, which in this case is 55kDa RARA. The results strongly indicate that the protein complexes banding at 70kDa may contain a RARA covalently modified by one SUMO-2 and, at 150kDa, it may contain RARA modified by multiple SUMO-2s in COS-7 cells.

Then, to determine whether RARA can be sumoylated by endogenous SUMO-2 in Sertoli cells, mouse Sertoli cell line (MSC-1 cells) were transfected with pFLAG-*RARA* cDNA construct and the protein lysate was prepared. Endogenous SUMO-2 was expressed in primary Sertoli cells, MSC-1 and COS-7 cells (Fig. 3.10, B). The Western blot (Fig. 3.3A) revealed intense higher molecular weight complexes, banding around 150kDa, in the lane with the lysate from the pFLAG-*RARA* cDNA transfected cells compared to the intensity of the same complexes in the lane with the lysates from the empty vector-transfected cells. More interestingly, *at*RA treatment of untransfected MSC-1 cells significantly enhanced

the intensity of ladder-like high molecular weight complexes (Fig. 3.3B), recognized by both anti-RARA and anti-SUMO-2/3 antibodies, which included the 150kDa and 70kDa complexes. Moreover, we found that primary Sertoli cells, without any transfections, but with a TCA concentration step, have the ladder-like pattern of higher molecular complexes recognized by anti-RARA and anti-SUMO-2/3 antibodies, further enhanced by retinoic acid and/or follicle stimulating hormone treatment (Fig. 3.3C), similar to those observed in MSC-1 cells (Fig. 3.3B). These results together suggest that sumoylation of endogenous RARA occurs in Sertoli cells and *at*RA can enhance sumoylation of endogeneous RARA in Sertoli cells.

SUMO-2 and *at*RA regulate the steady-state levels of sumoylated RARA and unconjugated RARA

Since human patients with hypo-sumoylation or hyper-sumoylation by SUMO-2/3 were found to have azoospermia and hypospermatogenesis (22), we varied the ratio of SUMO-2 to RARA to investigate the effect of endogenous, an intermediate, or a higher concentration of SUMO-2 on RARA sumoylation. Thus, COS-7 cells were cotransfected with a set amount of pFLAG-*RARA* and increasing amounts of pHis-*Sumo-2* cDNA constructs to determine the role of SUMO-2 on RARA. As expected, the level of SUMO-2/3 monomer was increased with the increasing amount of pHis-*Sumo-2* cDNA transfected into COS-7 cells (Fig. 3.10, C). In the absence of *at*RA treatment, sumoylated RARA was detected even at the endogeneous level of SUMO-2, albeit at a very low level, but steadily increased with the increasing concentration of SUMO-2 (Fig. 3.4A and B). However, *at*RA greatly enhanced SUMO-2 conjugation of RARA, even at the endogenous level of SUMO in COS-7 cells (compare Fig. 3.4A, lane 1 and Fig. 3.4C, lane 1), similar to MSC-1 cells (Fig. 3.3B). More interestingly, *at*RA modulated the amount of RARA and sumoylated RARA complexes in a biphasic fashion (Fig. 3.4D). The unconjugated RARA and sumoylated RARA started high at the endogenous level of SUMO-2, then as the SUMO-2 level increased, unconjugated RARA and sumoylated RARA declined, followed by an increase in the levels of RARA and sumoylated RARA at high concentrations of SUMO-2 (Fig. 3.4C and D).

Sumoylation of RARA requires proteasome function and new protein synthesis

To determine if an inhibitor to proteasomes can block the decline of sumoylated RARA at intermediate levels of SUMO-2, COS-7 cells were cotransfected with pFLAG-*RARA* and increasing amounts of pHis-*Sumo-2* cDNAs, pre-treated with MG132, a reversible proteasome inhibitor, for 30 min, and then treated with either vehicle or *at*RA and MG132 for 24 hrs. Unexpectedly, MG132 abolished the *at*RA-mediated biphasic effect on the sumoylation pattern (compare Fig. 3.4F and Fig. 3.4C). Similarly, no SUMO-2 conjugated RARA complexes were detected after MG132 treatment in absence of *at*RA (Fig. 3.4E). In contrast, MG132 seems to have a minimal influence at higher levels of SUMO-2 in the presence of *at*RA (Fig. 3.4F). Together, these results suggest that proteasomal function is required for RARA sumoylation at a concentration close to the endogenous level of SUMO-2, whereas at a higher level of SUMO-2, the proteasomal function is not necessary for sumoylation. These results are consistent with previously reported results showing that ubiquitin-mediated proteasome system is required for the recycling of SUMO-2/3 (23) and the transactivation activity of the mineralocorticoid receptor is controlled by sumoylation and proteasomal activities (24). In addition, COS-7 cells were transfected with pFLAG-*RARA* and an increasing amount of pHis-*Sumo-2* cDNA constructs, and pre-treated with cyclohexamide (CHX) for 30 min and with both *at*RA and CHX for 24 hrs to determine the role of new protein synthesis on sumoylation. Cyclohexamide virtually abolished *at*RA effect on the sumoylation pattern (Fig. 3.4G), suggesting that new protein synthesis is also required for SUMO-2 conjugation of RARA.

Mutations of SUMO-2 acceptor lysines influenced the steady-state levels of RARA and sumoylated RARA

To determine whether K399 in the ligand-binding domain of RARA could be a SUMO-2 acceptor lysine, K399 was mutated to arginine (R) to maintain the net positive charge. Then, COS-7 cells were cotransfected with pFLAG-*RARA* K399R and an increasing amount of pHis-*Sumo-2* cDNA constructs, and treated with either vehicle or *at*RA for 24 hrs. The K399R mutation in RARA totally abrogated the

appearance of SUMO-2 conjugated RARA complexes in the absence of *at*RA (Fig. 3.5A). This indicates that K399 is the sole site of sumoylation in the absence of *at*RA, albeit only a small percentage of RARA is sumoylated at the endogenous level of SUMO-2 (Fig. 3.4A). However, in the presence of *at*RA, the expression level of RARA K399R and the associated SUMO-2 conjugated RARA complexes were enhanced with an increasing level of SUMO-2 (Fig. 3.5B). The results suggest that, upon *at*RA binding, the conformation of RARA changes, as previously shown (25), and then K147, K166, K171 SUMO-2 acceptor sites may be revealed and modified by SUMO-2. Moreover, the results also suggest that the ligand binding and the subsequent conformational change of the receptor are not affected by the K399 mutation.

To ascertain whether K147 in the DNA binding or K166 and K171 in the nuclear localization domains are subjected to sumoylation in the presence of *at*RA, cDNA constructs were generated to substitute lysine with arginine at these positions and in combination with the K399R mutation. Triple and quadruple mutants (K166/171/399R and K147/166/171/399R) were generated. COS-7 cells were cotransfected with pHis-*Sumo-2* cDNA and either pFLAG-*RARA* (WT) or the different pFLAG-*RARA* mutant cDNAs, and then treated with either vehicle or *at*RA. The RARA K171/399R and RARA K166/399R (Fig. 3.5C, lanes 6 and 8) displayed the lowest and next lowest intensities of SUMO-2 conjugated RARA complexes among the double mutants, respectively. This suggests that K171 and K161 are the first and second SUMO-2 acceptor sites in the presence of *at*RA, respectively. Interestingly, we also note that RARA K147/399R displayed even higher intensity of the SUMO-2 conjugated RARA complexes (Fig. 3.5C, lane 4) than that of the wild type (Fig. 3.5C, lane 2), indicating that K166 and K171 sumoylation alone results in the maximum level of sumoylated RARA complexes in the presence of *at*RA.

Sumoylation of RARA at specific SUMO-2 acceptor sites influenced the subcellular localization of RARA

To determine whether mutations at K399 and other lysine acceptor sites influence the nuclear

localization of the receptor at the endogeneous level of SUMO-2 (Fig. 3.6) and at over-expressed SUMO-2 levels (Fig. 3.11), COS-7 cells were transfected with pFLAG-*RARA* (WT), various mutant cDNA constructs, or if applicable, SUMO-2 cDNA, and treated with either vehicle or *at*RA for 30 min, and then subjected to indirect immunofluorescence assay using anti-FLAG M2 antibody. Surprisingly, totally opposite from expected for RARA WT (Fig. 3.6A), RARA K399R was found ~67% of cells in the nucleus, in the absence of *at*RA (Fig. 3.6B). Even more interestingly, in the absence of *at*RA, the nuclear localization of the double, the triple and the quadruple mutant of RARA, which all included the mutated K399 site, had a nuclear localization pattern (Fig. 3.6E, F, G and H) similar to that of RARA K399R mutant (Fig. 3.6B). In contrast, RARA K166R and RARA K171R with only single site mutated had a cytoplasmic localization pattern (Fig. 3.6C and D), similar to that observed for RARA WT in the absence of *at*RA (Fig. 3.6A). These data suggest that, in the absence of *at*RA, K399 in the ligand-binding domain of RARA plays a key role in maintaining RARA in the cytoplasm.

Strikingly, in the presence of *at*RA, RARA K399R was observed in ~75% of cells in the cytoplasm (Fig. 3.6J), which was again completely opposite from that of RARA WT (Fig. 3.6I) and other single mutants of RARA (Fig. 3.6K and L). This suggests that sumoylation at K166 and K171 inhibits RARA nuclear localization, if K399 is not functional. Functional K399 is necessary for *at*RA-control of nuclear trafficking, but it is not because *at*RA does not bind to the K399R mutated receptor. On the other hand, the triple mutant with K166, K171 and K399 mutated and the quadruple mutant were primarily observed in the nucleus in the presence of *at*RA (Fig. 3.6O and P) or in the absence of *at*RA (Fig. 3.6G and H). These results together suggest that, when all three sites are mutated, RARA is constitutively found in the nucleus and cannot be regulated by *at*RA.

High level of SUMO-2 interferes with the transcriptional activity of RARA

To determine whether sumoylation has an effect on the transcriptional activity of RARA, COS-7 cells were transfected with pFLAG-*RARA*, pcDNA-*Rxra*, pcDNA-*β-gal,* pRARE-tk-*Luc* reporter construct along with increasing amounts of pHis-*Sumo-2*, and the luciferase activity assayed to determine the

transcriptional capacity. The highest luciferase activity was detected at the endogenous level of SUMO-2, whereas the transcriptional activity of RARA declined with increasing SUMO-2 expression (Fig. 3.7A). This repression did not come from the decreased amount of RARA, because the steady-state level of RARA increased at the highest level of SUMO-2 (Fig. 3.4C). The suppression of transcriptional activity was observed previously for covalently sumoylated transcription factors such as with c-Jun, c-Myb, and NF*k*B (26-28). To eliminate the possibility that RARE could be recognized by other nuclear receptors such as RARB or RARG (2, 4), we performed the same experiment, but treated the cells with Am580, a specific RARA agonist. Similar results were obtained with Am580 (Fig. 3.7B), indicating that the changes in the luciferase activity were generated by RARA-specific transcriptions. Therefore, these results demonstrate that over-expressed SUMO-2 can specifically suppress the transcriptional activity of RARA.

Mutations in K166, K171, and K399 enhanced the transcriptional activity of the mutant RARA

To determine if SUMO-2 acceptor lysine substitution to arginine have any effect on the transcriptional activity of RARA, COS-7 cells were transfected with either pFLAG-*RARA* (WT) or mutant RARA cDNA, pcDNA-*Rxra* cDNA, pRARE-tk-*Luc* cDNA, and pcDNA-*β-gal* cDNA plasmids. In the presence of *at*RA, a mutation at K399 of RARA caused a significant decrease in its transcriptional activity, compared to that of RARA WT (Fig. 3.7C). The other single mutants, with K166R or K171R mutations, also displayed a significantly reduced level of transcriptional activity, compared to that of RARA WT (Fig. 3.7C), but the level was also significantly higher than that of the RARA K399R. In contrast, the transcriptional activity of RARA K166/399R, RARA K171/399R, the triple mutant and the quadruple mutant were as high as or significantly higher than that of RARA WT (Fig. 3.7D), indicating that sumoylation on either K166 or K171 could suppress the transcriptional activity of RARA. Taken together, these results demonstrate that a functional K399 site is required for the wild-type transcriptional activity of RARA, and sumoylation on K166 and K171 sites could suppress the transcriptional activity of RARA.

SENPs may be responsible for editing and de-conjugating SUMO-2 from RARA prior to transcriptional activity

To determine whether sumoylation on K166 and K171 could be removed prior to transcriptional activity, we determined the mRNA levels of *Senp'*s from primary Sertoli cell mRNAs, germ cells, testes from postnatal day 10 (P10), and from adults by realtime PCR. Some SENPs are involved in proteolytic processing of SUMO precursors, some only de-sumoylate or some can de-sumoylate and edit the poly-chain (29). In addition, SENPs are located specifically in the cytoplasm, the nuclear side of nuclear pore complex or located in the nucleoplasm (30). We found that *Senp6*, *Senp3, and Senp1*, in this order, were highly expressed in Sertoli cells (Fig. 3.8A), whereas *Senp3* was the dominant one in germ cells (Fig. 3.8B). Consistently, *Senp3* was the highest in adult testis (Fig. 3.8C), which contains of more germ cells than Sertoli cells, while both *Senp3* and *Senp6* were found in testis from P10 rats (Fig. 3.8D), reflecting the higher ratio of Sertoli cells to germ cells than in adults. Interestingly, it is reported that SENP1 is localized to the nuclear pore and works as processing and deconjugation enzyme while SENP3 is localized predominately in the nucleolus and serves as an enzyme that deconjugates SUMO-2/3 from substrates (16). SENP6 is the largest SENP, containing multiple nuclear localization domains, and is found in the cytoplasm and the nucleoplasm (16), specific for editing SUMO-2/3 from a poly-SUMO-2/3 chain (29). Thus, SENP6 and SENP3 could be responsible for de-conjugating SUMO-2/3 from K166 or K171 sites of RARA in Sertoli cells.

3.4 Discussion

Recently, sumoylation has joined the list of post-translational modification mechanisms proven to be important in regulating the dynamic nuclear localization and transcriptional functions of nuclear receptors (10-14, 31, 32). Previously, SUMO-1 was found to covalently link to RARA in the presence of *at*RA (33), however, the role of SUMO-2/3, that can poly-sumoylate, on RARA has not been investigated. Ectopic expression of *RARA* and *Sumo-2* cDNAs in COS-7 cells significantly enhanced the amount of the protein

complexes around 150kDa, indicating explicitly that the RARA-immunoreactive higher molecular complexes consisting of RARA, derived from *RARA* cDNA, were covalently modified by multiple SUMO-2s (Fig. 3.2A and B).

Interestingly, *at*RA enhanced higher molecular weight RARA complexes in untransfected Sertoli cells (Fig. 3.3B and C) as well as in COS-7 cells that were transfected with *RARA* cDNA (Fig. 3.4C), suggesting that *at*RA is an active inducer of RARA sumoylation in Sertoli cells and COS-7 cells. In fact, the pattern of sumoylation was biphasic in the presence of *at*RA (Fig. 3.4C and D). Retrospectively, we should have anticipated the decline of RARA and sumoylated RARA, in the presence of *at*RA, because it is known that *at*RA triggers RARA to undergo degradation by the ubiquitin-mediated proteasome pathway immediately after the transcription of its target gene (13). In a similar manner, an increase of RARA and sumoylated RARA could be due to over-expressed SUMO-2 inhibiting the transcriptional activity of RARA (Fig. 3.7A and B), and thus making RARA inaccessible to transcription-associated ubiquitin-mediated proteasome system. Alternatively, when SUMO-2 is over-expressed, we cannot eliminate the possibility that cofactors could be sumoylated. The cofactors such as steroid receptor coactivator 1 (SRC1), histone deacetylase 1 (HDAC1), histone deacetylase 4 (HDAC4) and p300 have been shown to be modified by SUMO-1 and repress the activity of target genes (34-36). Nonetheless, these molecular mechanisms could easily serve as the underlying basis of azoospermia and hypospermatogenesis in human patients with hyper-sumoylation by SUMO-2/3 (22).

Site-directed mutational studies showed that RARA is modified by SUMO-2 on K399 in the absence of *at*RA (Fig. 3.5A and 5B). Remarkably, the banding pattern was similar to the one in which cells were not treated with *at*RA (Fig. 3.4A), suggesting that the *at*RA-mediated biphasic sumoylation pattern for the RARA WT (Fig. 3.4C and D), is dependent on a functional K399 site. Additional mutational studies provided evidence that RARA is sumoylated primarily on K171 and then on K166, in this order, in the presence of *at*RA (Fig. 3.5C, lanes 8 and 6). This has a precedence, reported for sumoylation of androgen receptor on K511, which was agonist dependent, whereas K318 sumoylation was not (37). Nuclear localization studies revealed unexpected results that RARA K399R was found in the nucleus, in the

absence of *at*RA (Fig. 3.6B), while RARA K399R was found in the cytoplasm, in the presence of *at*RA (Fig. 3.6J). These results strongly provide evidence that a functional K399 of RARA plays a key role in the *at*RA-mediated regulation of the receptor subcellular localization. Consistently, K399R mutation played a dominant role in the absence of *at*RA, such that double, triple, and quadruple mutants that included K399R mutation all remained in the nucleus (Fig. 3.6E, F, G and H).

On the other hand, the fact that RARA K399R was found in the cytoplasm, in the presence of *at*RA (Fig. 3.6J), suggests that K171 and K166 loaded with SUMO-2, without a functional K399 site, may be inhibitory to nuclear localization. Supportive of this, the triple mutant RARA K166/171/399R was in the nucleus constitutively (Fig. 3.6G and O), in effect, escaping *at*RA-mediated regulation of subcellular localization. Consistent with the subcellular localization results, RARA K399R had only a basal level transcriptional activity, while RARA K166/171/399R had even higher transcriptional activity than that of RARA WT, indicating that sumoylation on K166 and K171 has an inhibitory role in regulating the transcriptional activity of RARA (Fig. 3.7D).

Interestingly, the high expression of SENP6 in Sertoli cells indicates that it could be responsible for desumoylation in the cytoplasm and it could also move with the receptor into the nucleus since it has multiple nuclear localization signals. In other words, the nuclear localization process and successful transcriptional activity of the RARA are accomplished if a coordinated sumoylations and desumoylations of RARA, occurs, with the desumoylation conducted by SENPs, especially SENP6 in Sertoli cells. Previously, SENP6, also known as SUSP1, has been shown to co-localize with RXRA and remove SUMO-1 from RXRA to mediate the transcriptional activity of RXRA-RARA complex (38). Thus, we propose a working model of mechanisms (Fig. 3.9A), where a functional K399 is critical for the *at*RA-mediated control of nuclear localization and the processing of sumoylation on K171 and K166 (Fig. 3.9A). SENP6 could be bound to the K399 site of the RARA in the absence of *at*RA and keep sumoylation level low and then in the presence of *at*RA, SENP6 accompany RARA into the nucleus and desumolyate K171 and K166 sites along the way, before RARA is used as a transcription factor. When K399 site is mutated, SENP6 is not bound, but K171 and K166 are sumoylated in the presence of *at*RA and then the receptor is

in the cytoplasm, whereas in the absence of *at*RA, the receptor is like the triple mutant without any sumoylations. When all sites are mutated, the K399 site is not able to control the nuclear localization process and thus the receptor can be in the nucleus constitutively (Fig. 3.9B), resulting in the highest transcriptional activity (Fig. 3.7D). However, when hyper-sumoylation occurs (Fig. 3.9C), SENP enzymes may not have enough capacity to take off SUMO-2 from RARA, resulting in accumulation of sumoylated RARA in the nucleus and in the cytoplasm (Fig. 3.11). Sumoylated RARA may work as a dominant negative receptor blocking unsumoylated RARA from binding to its partner RXR, and impeding the transcription and degradation of unsumoylated RARA (Fig. 3.9C). These mechanisms require further investigation in the future.

In conclusion, we have identified SUMO-2 modification of RARA as a novel post-translational modification for RARA. The *at*RA and the SUMO-2 are two key factors regulating sumoylation of RARA. Sumoylation affects the stability of RARA and SUMO-2 conjugated RARA, influences the nuclear localization of RARA, and modulates the transcriptional activity of RARA. Mutational studies have elucidated potential mechanisms of sumoylation and desumoylation for regulating *at*RA-mediated nuclear localization and transcriptional activity. Thus, manipulation of SUMO-2 could be an alternative strategy to control the function of RARA and hypo- or hyper SUMO-2 modification could be detrimental to proper function of RARA.

3.5 Materials and Methods

Plasmid Constructs

A *hRARA* cDNA from LRARaSN (39) was subcloned into the pFLAG-CMV2 vector (Sigma-Aldrich Co., St Louis, MO) using *Eco*RI and *Bam*HI. To mutate specific residues in RARA, the QuickChange™ Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) was used. The entire resulting cDNAs were sequenced to confirm the site-directed mutations. pFLAG-*Sumo-2* and pHis-*Sumo-2* cDNAs were constructed by inserting the full-length rat *Sumo*-2 cDNA into pFLAG-CMV-2 and pQE-TriSystem, respectively (Qiagen Inc., Valencia, CA).

Antibodies and reagents

An affinity-purified rabbit polyclonal antibody raised against a full-length SUMO-2 protein recognized both SUMO-2 and 3 (Zymed Laboratories, Inc., San Francisco, CA). Anti-FLAG M2 monoclonal antibody (Sigma-Aldrich), rabbit anti-actin antibody (Sigma-Aldrich) and an affinity-purified rabbit polyclonal anti-RARA antibody, raised against a human RARA peptide consisting of amino acids 443-462 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), were utilized. Cycloheximide (CHX) that inhibits new protein synthesis (Sigma-Aldrich), carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132), a reversible, cell permeable proteasome inhibitor (EMD Chemicals Inc., Gibbstown, NJ), and Am580, a RARA-specific agonist (Biomol Research Laboratories, Plymouth Meeting, PA), were purchased. Recombinant human follicle stimulating hormone (FSH) was from Dr. A. F. Parlow, the National Hormone and Peptide Program.

Cell culture

MSC-1 and COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing either 5% or 10% fetal bovine serum, respectively, supplemented with penicillin (100 IU/mL) and streptomycin (100 μg/mL) at 37 ^{o}C. Primary Sertoli cells were isolated from the testes of 20-day-old rats by sequential enzymatic digestions, as previously described (11). Sertoli cells were plated under serum-free conditions in Ham's F-12 medium (Invitrogen Laboratories, Carlsbad, CA) and cultured in 32 ^{o}C for up to 72 hrs. Animal protocols, following the NIH guidelines, were approved by the Institutional Animal Care and Use Committee of Washington State University. Germ cells from four adult male rat (~60 days of age) were isolated using collagenase and trypsin as previously described (40). The percentage of germ cells in each preparation was determined. The cells were smeared onto a slide, fixed with Bouin's solution for 1 hr, and stained with hematoxylin, and the number of germ cells was determined. More than 200 cells per preparation were counted. The average percentage of germ cells in three separate germ cell preparation was 84.3% ± 0.7 (mean ± SD).

Concentration of immunoprecipitated proteins from Sertoli cells

Primary Sertoli or MSC-1 cells from three 150 mm plates were lysed in a buffer containing 10 mM Tris-HCl (pH 8.0), 10 mM KCl, 1.5 mM $MgCl_2$, and a cocktail of protease inhibitors (Roche Diagnostics, Indianapolis, In). After 20 min on ice, cell were centrifuged at 750 g for 5 min, the supernatant was saved as the non-nuclear fraction. The pellet was lysed in five packed-volumes of cold nuclear lysis buffer consisting of 50 mM Tris-HCl (pH 8.0), 420 mM KCl, 5 mM $MgCl_2$, 100 mM EDTA (pH 8.0), 20% glycerol, 10% sucrose, and protease inhibitors. After pre-clearing, the lysate was loaded on a column packed with anti-RARA antibody cross-linked to protein A agarose beads (Sigma-Aldrich) and incubated for 3 hrs. Antibody was cross-linked to agarose beads by incubation with dimethylpimelinidate triethlyamine. The column was washed with a buffer containing 50 mM Tris-HCl (pH 8.0), 500 mM LiCl, 1 mM EDTA (pH 8.0), 10% glycerol, followed by a buffer containing 10 mM HEPES (pH 7.4), 5 mM NaCl, 1 mM EDTA (pH 8.0), 10% glycerol. Bound proteins were eluted with 20 mM triethlyamine and 10% glycerol. To concentrate the eluted proteins, 1/4 volume of ice-cold trichloroacetic acid (TCA) (100% w/v) and 4 mg/ml sodium deoxycholate were added, incubated on ice for 30 min, followed by centrifugation at 16,000 g for 10 min at 4 ^{o}C. The pellet was washed with acetone (-20 ^{o}C), incubated on ice for 10 min, and then centrifuged 16,000 g for 10 min at 4 ^{o}C. The final pellet was air-dried and resuspended in two packed-volumes of SDS-PAGE loading buffer for Western blot analysis.

Western blot analysis

Western blot analyses were performed as described previously (11). Proteins were subjected to SDS-PAGE and transferred to Immobilon-P membranes (Millipore Co., Bedford, MA). After blocking with 5% non-fat milk in tris-buffered saline with 0.5% Tween (0.5% TBST) for 1 hr, membranes were incubated with primary antibody and then with horseradish peroxidase-conjugated secondary antibody both in 0.5% TBST for 1 hr at room temperature (RT). Equal loading was determined using Commassie blue dye-stained membrane or Western blot analysis with anti-actin antibody. Blots were processed with

Enhanced Chemiluminescence Western blotting system (Amersham Biosciences, Piscataway, NJ).

Transient transfection and SUMO-2 and RARA co-immunoprecipitation analysis

75% confluent COS-7 cells in 60 mm plates were transfected with 1:1 ratio (6 μg total per plate) of pFLAG-*RARA* and pFLAG-*Sumo-2* cDNA constructs using lipofectamine 2000 reagent (Invitrogen Laboratories). 48 hrs after transfection, cells were lysed in modified RIPA buffer (50 mM Tris/HCl, pH7.4, containing 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 10% SDS, 1 mM dithiothreitol and a protease-inhibitor) and the lysates were pre-cleared by incubation with True Blot Protein G Sepharose beads (eBiosciences, Inc., San Diego, CA) for 1 h at 4 ^{o}C. After centrifugation at 10,000 g at 4 ^{o}C, the supernatant was immunoprecipitated with anti-RARA antibody or anti-SUMO-2/3 antibody with Protein G Sepharose beads. Immunoprecipitates were washed extensively and analyzed by Western blotting technique with an anti-RARA antibody or anti-SUMO-2/3 antibody.

Transient transfection, immunoblotting, and luciferase reporter assay

75% confluent MSC-1 and COS-7 cells in 6-well or 24-well plates were transfected with various cDNA constructs. The luciferase reporter plasmid, pRARE-tk-*Luc*, which contains three RAREs from the *RARB* promoter, and pcDNA-*β-gal,* used for transfection efficiency, were described previously (10). In all cases, the total amounts of DNA were adjusted to 3 μg per well for a 6-well plate and 1 μg per well for a 24-well plate using the empty vector plasmid, pcDNA-3.1(-) (Invitrogen). Transfected cells were allowed to recover for 24 hrs and then cultured for additional 24 hrs, during which time, if needed, the cells were treated with appropriate reagents. After that, cells were lysed in modified RIPA buffer, incubated on ice for 10 min, and centrifuged at 16,000 g for 10 min at 4 ^{o}C to remove cell debris. For immunoblotting, the supernatant containing 50 μg of proteins were subjected to Western blot analysis. Protein concentration was determined by the Bradford assay with BSA as the standard. For luciferase reporter assays, the supernatant was assayed for luciferase activity using a Luciferase Assay System (Promega Co., Madison, WI). The *β*-galactosidase activity was determined with the Galactosidase Assay

System (Promega).

Transient transfection and indirect immunofluorescence assay

COS-7 cells, seeded on coverslips in 24-well plates, were transfected with various cDNA constructs. After 48 hrs post-transfection, cells were treated with various reagents for 30 min and fixed in -20 ^{o}C methanol for 10 min. Cells were blocked with 10% normal goat serum (Vector Laboratories, Burlingame, CA) for 1 h, before incubation with anti-FLAG M2 antibody overnight at 4 ^{o}C. Cells were washed with PBS and incubated with a biotinylated secondary antibody at 1:300 dilutions for 1 hr at RT. Detection of antibody complexes was conducted using fluorescein avidin D (Vector Laboratories). All digital images were obtained using a laser confocal system (Zeiss LSM 510, Hitachi, Japan).

RNA extraction and real-time (quantitative) RT-PCR

Total RNA from isolated Sertoli cells, germ cell, 10-day-old and 60-day-old rat testes was collected using TRIzol regent (Invitrogen). Real-time PCR primers for each *Senps* were designed using Primer Express software version 2.0 (Applied Biosystems). The primers used to amplify each specific gene were listed (Supplemental Table 1). Complementary DNA was synthesized from 500 ng RNA samples using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Subsequently, 20 ng cDNA was used as template for real-time PCR assays with a Gene Amp 7000 thermocycler (Applied Biosystems). Threshold (Ct) values for *Senps* and the housekeeping gene *S2* were determined using Prism SDS software version 1.2 (Applied Biosystems). The level of *Senp*s real-time PCR product was normalized to that for *S2* real-time PCR product in each sample. The real-time PCR was conducted on three samples of cDNAs in triplicate.

Statistical analysis

Statistical analysis consisted of one-way ANOVA, followed by a pairwise comparison of the means at α=0.05 (Tukey-Kramer method, JMP; SAS Institute Inc., Cary, NC).

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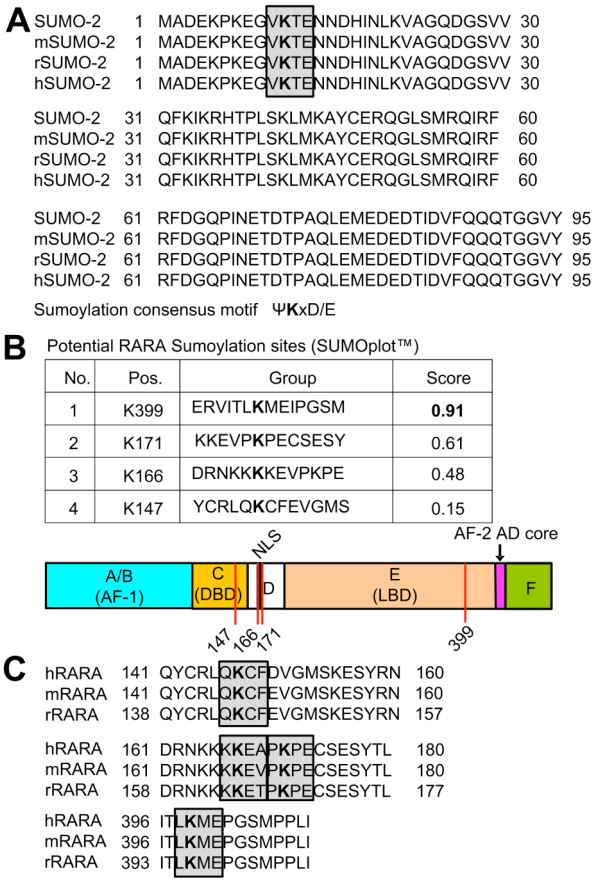

FIG. 3.1. **RARA has SUMO acceptor sites**. A, The translated protein sequence of *Sumo-2* cDNA isolated from a LacZ-positive yeast clone, identified as containing a protein that strongly interacts with RARA. The SUMO-2 sequence was identical to mouse (mSUMO-2), rat (rSUMO-2), and human (hSUMO-2) sequences. The potential sumoylation sites are boxed and the consensus sequence is indicated. B, Four potential sumoylation sites of RARA, K399, K171, K166 and K147, predicted by SUMOplot^{TM} software, arranged according to predicted score from high to low. Schematic of the *hRARA*, showing K399 in LBD, K166 and K171 in the NLS, and K147 in the DBD. C, Sequence alignment of sumoylation sites (boxed) and surrounding sequences of RARA from human (hRARA), mouse (mRARA), and rat (rRARA)

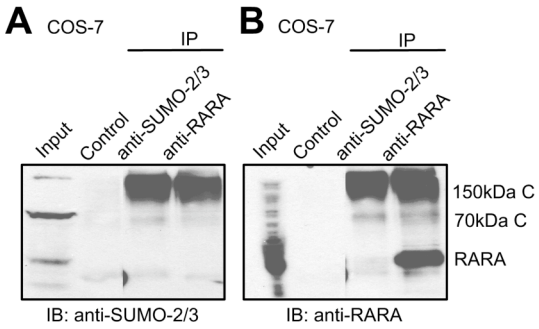

FIG. 3.2. **RARA is covalently modified by SUMO-2 in COS-7 cells.** A-B, COS-7 cells were transiently transfected with pFLAG-*RARA* cDNA and pFLAG-*Sumo-2* cDNA constructs, lysed, immumoprecipitated (IP) with either anti-RARA or anti-SUMO-2/3 antibodies, followed by immunoblotting (IB) with anti-SUMO-2/3 (A) or anti-RARA (B) antibody. Input, lysate used for immunoprecipitation; Control, IgG beads incubated with the lysate only. 150 kDa C, protein complexes around 150kDa. All experiments were repeated at least three times.

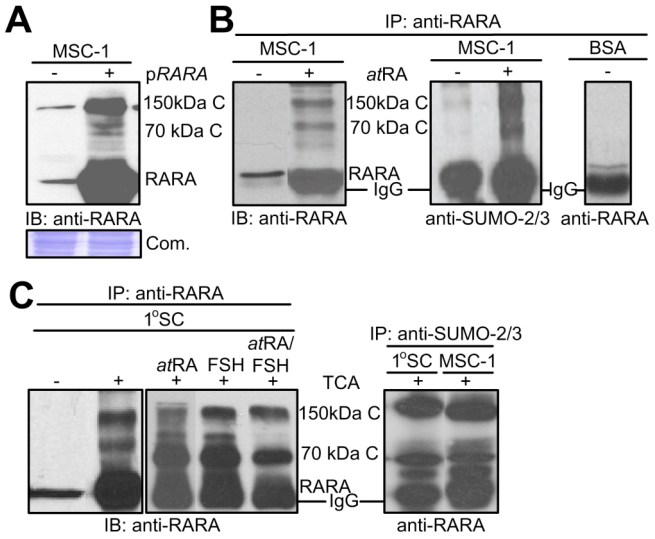

FIG. 3.3. **RARA is covalently modified by SUMO-2 in MSC-1 and primary Sertoli cells.** A, MSC-1 cells, transfected with either empty vector or pFLAG-*RARA* cDNA (p*RARA*) construct, were lysed and subjected to immunoblotting (IB) with anti-RARA antibody. Equal loading was determined by the Commassie blue dye stained membranes (Com.). B, MSC-1 cells were treated with either vehicle or *at*RA (1 μM), lysed after 3 hrs, loaded onto a column with anti-RARA antibody cross-linked to protein A agarose beads (IP). The eluate was TCA precipitated and immunoblotted with anti-RARA or anti-Sumo-2/3 antibody. BSA was loaded on a column packed with anti-RARA antibody cross-linked to protein A agarose beads and immunoblotted (IB) with anti-RARA, as a negative control. C. Nuclear extracts of primary Sertoli cells or MSC-1, treated with *at*RA (1 μM), or FSH (25 ng/ml), or *at*RA and FSH, were loaded onto a column with anti-RARA antibody or anti-Sumo-2/3 antibody cross-linked to protein A agarose beads (IP), the eluate collected (-), TCA precipitated (+), and immunoblotted (IB) with anti-RARA antibody. 150 kDa C, protein complexes around 150kDa; IgG, IgG contaminant in the eluate from the large-scale IP. All experiments were repeated at least three times.

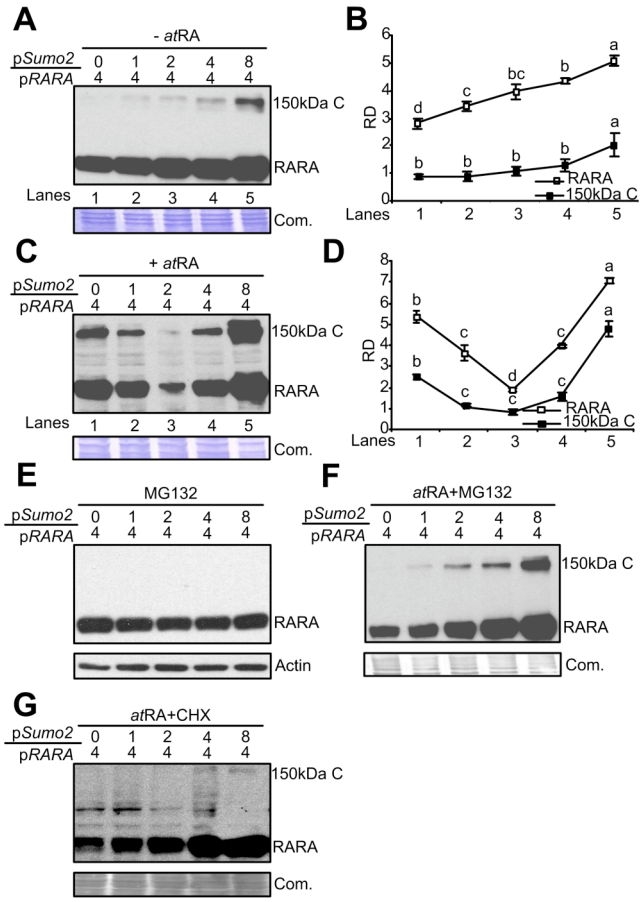

FIG. 3.4. **SUMO-2 and *at*RA regulate the steady-state levels of sumoylated RARA and unconjugated RARA, but sumoylation requires functional proteasomes and new protein synthesis.** A-G, COS-7 cells were transfected with a varying ratio (0:4, 1:4, 2:4, 4:4, and 8:4) of pHis-*Sumo-2* (p*Sumo-2*) to pFLAG-*RARA* (p*RARA*) cDNA constructs, treated with either vehicle (A), *at*RA (1 μM) (C), MG132 (10 nM) (E), or pretreated with MG132 (F) or CHX (100 μg/mL) (G) for 30 min and then treated with *at*RA and MG132 (F) or *at*RA and CHX (G). Cells were lysed and immunoblotted with anti-FLAG M2 antibody. Equal loading was determined by the Commassie blue dye stained membranes (Com.) or anti-actin antibody. B and D, The intensities of RARA and sumoylated RARA in the absence (B) and presence (D) of *at*RA, represented in (A and C), were scanned and graphed with means ± SEM from n=3 independent experiments. The different letters denote a significant difference from each other at $p \leq 0.05$. RD, relative density; open square, RARA; solid square, 150kDa C. All the experiments were repeated at least three times.

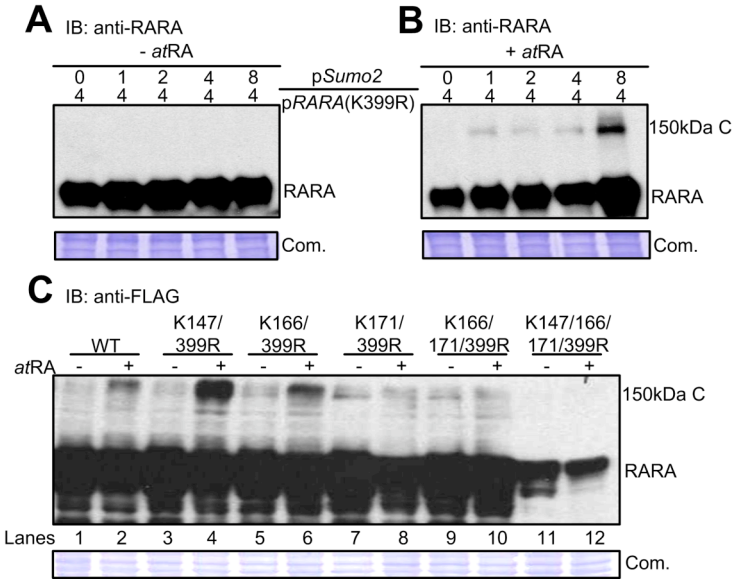

FIG. 3.5. **Mutations of SUMO-2 acceptor lysines influenced the steady-state levels of RARA.** A-C, COS-7 cells were transfected with a varying ratio (0:4, 1:4, 2:4, 4:4, and 8:4) of pHis-*Sumo-2 (*p*Sumo-2)* to pFLAG-*RARA*K399R (p*RARA*K399R)cDNA constructs (A and B) or a set amount of pHis-*Sumo-2 (*p*Sumo-2)* and pFLAG-*RARA* (WT) or mutant *RARA* cDNA constructs, 4:4 ratio (C). Cells were treated with either vehicle (A and -) or *at*RA (1 μM) (B and +), lysed and immunoblotted (IB) with anti-RARA antibody (A and B) or anti-FLAG M2 antibody (C). Equal loading was determined by the Commassie blue dye stained membranes (Com.). 150 kDa C, protein complexes around 150kDa. All the experiments were repeated at least three times.

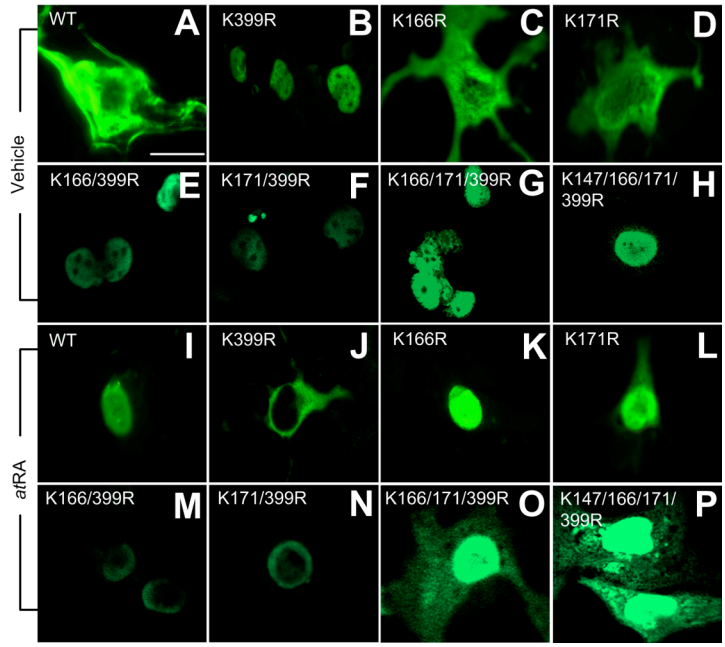

FIG. 3.6. **SUMO-2 modification of RARA at specific SUMO-2 acceptor lysines influences the nuclear localization of RARA.** COS-7 cells, transfected with the pFLAG-*RARA* (WT) or mutant *RARA* cDNA constructs, treated with either vehicle or *at*RA (1 μM) for 30 min, fixed, and analyzed by immunofluorescence with anti-FLAG M2 antibody. Bar in A = 20 μm, for A-P. The image shown represents at least ~65% of cells.

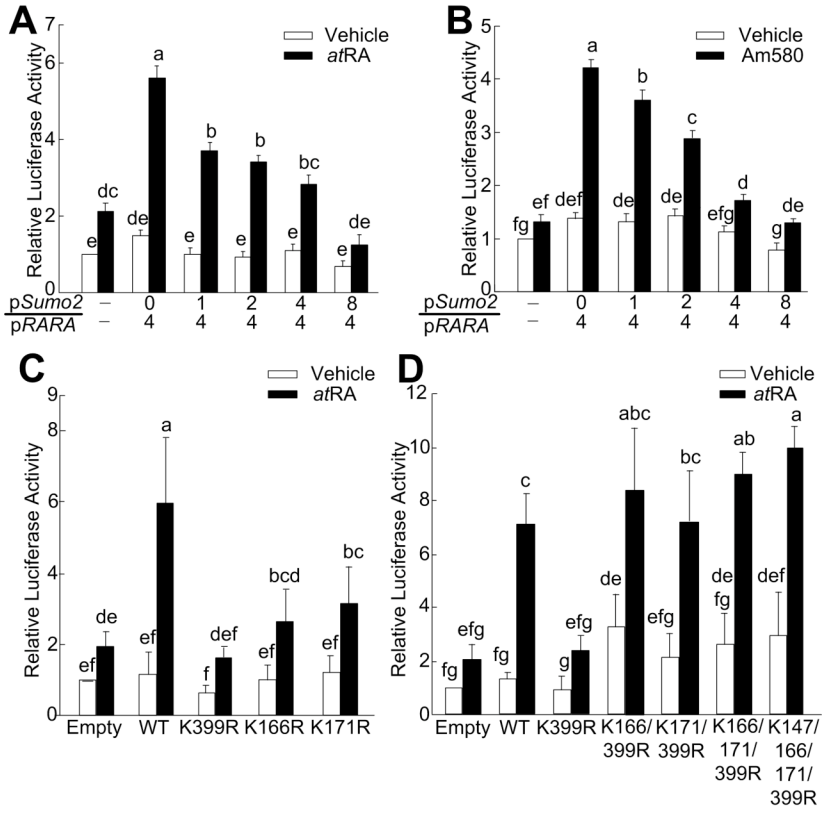

FIG. 3.7. **SUMO-2 modification of RARA at specific SUMO-2 acceptor lysines influences the transcriptional activity of RARA.** COS-7 cells were cotransfected with empty vector or a varying ratio (0:4, 1:4, 2:4, 4:4, and 8:4) of pHis-*Sumo-2 (*p*Sumo-2)* to pFLAG-*RARA* (p*RARA*) cDNA (A and B) or a set amount of pHis-*Sumo-2* and pFLAG-*RARA* (WT) and mutant *RARA* cDNA constructs (C and D), pcDNA-*Rxra* cDNA, pRARE-tk-*Luc* cDNA and pcDNA-*β-gal* cDNA constructs. Cells were treated with either vehicle (white bars) or *at*RA (1 μM) (A, C, and D) (black bars) or Am580 (0.1 μM) (B) (black bars), for 24 hrs and analyzed by luciferase reporter assay. Results were expressed as means ± SEM from nine replicates, n=3 independent experiments. Different letters denote a significant difference from each other at $p<0.05$. All the experiments were repeated at least three times.

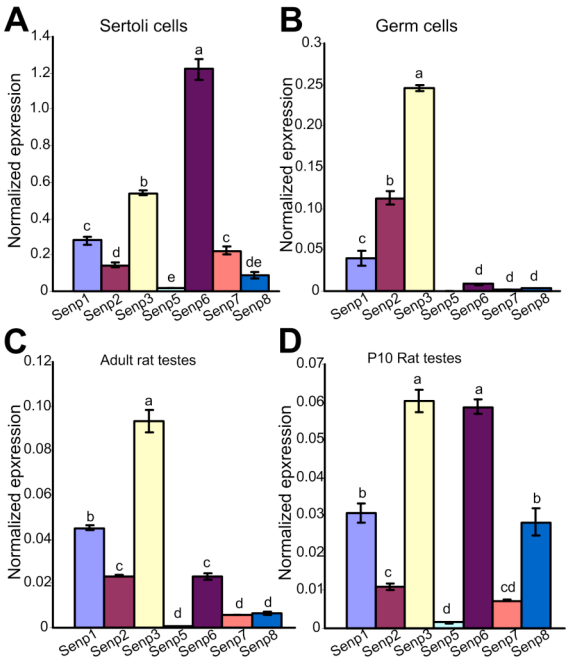

FIG. 3.8. **Expression profiles of the genes encoding the SUMO-specific proteases (*Senp*)**. A-D, Relative quantification of each *Senp* gene was determined in Sertoli cells (A), germ cells (B), adult rat testes (C) and testes from 10-day-old rats (D) using real-time PCR. Expression was normalized to the ribosomal protein *S2* expression. Results were expressed as means ± SEM from nine replicates, n=3 independent RNA samples. Different letters denote a significant difference from each other at $p<0.05$.

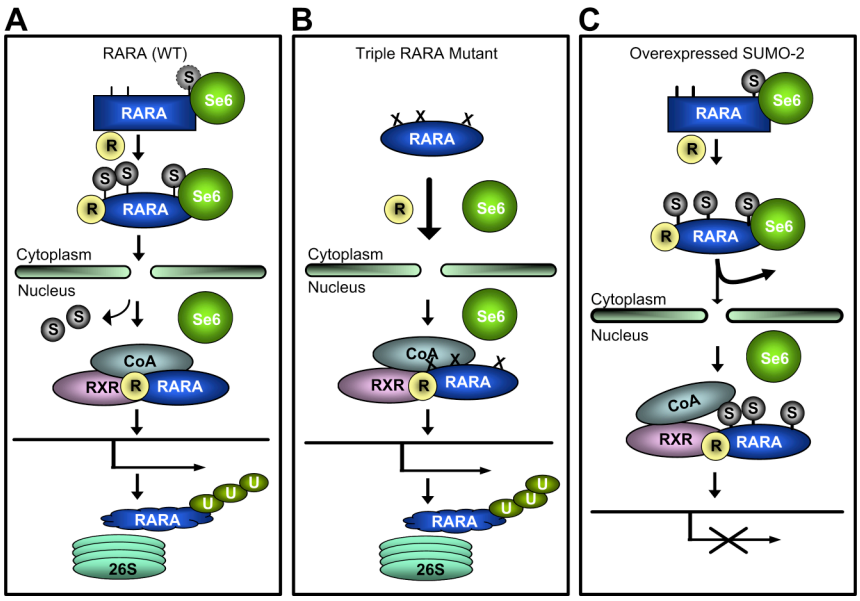

FIG. 3.9. **Models for RARA sumoylation, transcription, and degradation for the WT (A), for the triple mutant (B) and for overexpression of SUMO-2 (C).** Binding of *at*RA to RARA (rectangle) induces a conformational change to RARA (oval) that reveals K171 and K166 sumoylation sites. S, SUMO-2; Se6, SENP 6; CoA, coactivator; R, retinoic acid; tick marks represent K166, K171, and K399 sites; x marks represent K166R, K171R, and K399R mutations; RARA-U-U-U, ubiquitinated RARA; and 26S, proteasomes.

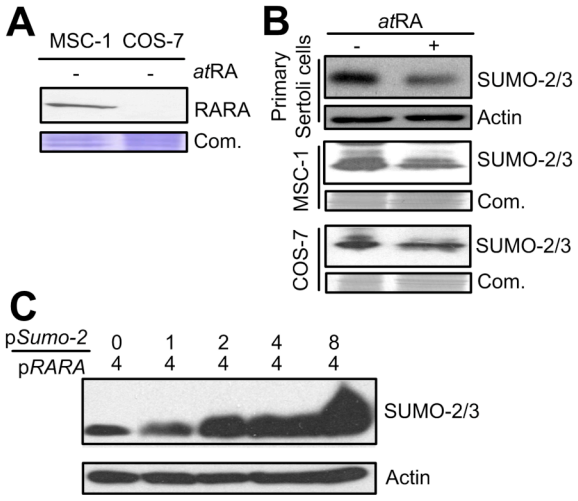

FIG.3.10. **Endogenous levels of RARA (A) and SUMO-2/3 (B) in primary Sertoli cells, MSC-1 and COS-7 and the increased levels of SUMO-2 after transfection.** A-B. Cells were treated with vehicle (-, A and B) or *at*RA (1 μ M) (+, B) for 24 h, lysed and subjected to immunoblotting (IB) with anti-RARA antibody (A) and anti-SUMO-2/3 antibody (B). Equal loading was determined by the Commassie stained membrane (Com.). C, SUMO-2 increased in a dose-dependent manner when COS-7 cells were transfected with a varying ratio (0:4, 1:4, 2:4, 4:4, and 8:4) of pHis-*Sumo-2* (p*Sumo-2*) to pFLAG-*RARA* (p*RARA*) cDNA constructs, lysed, and immunoblotted with anti-SUMO-2/3 antibody.

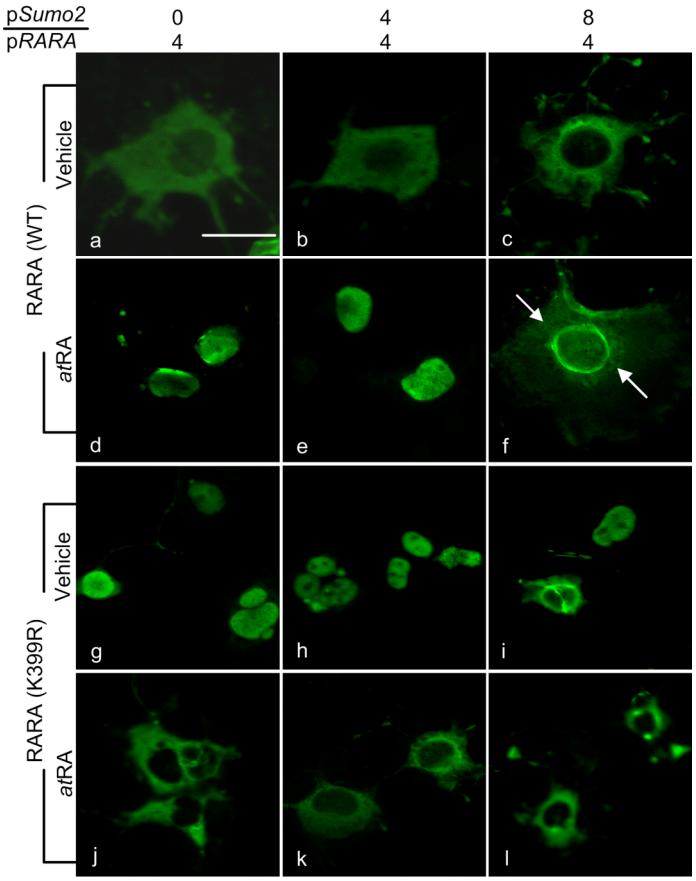

FIG. 3.11. **Subcellular localization assay in the presence of SUMO-2 overexpression.** COS-7 cells were transfected with a varying ratio (0:4, 4:4, and 8:4) of pHis-*Sumo-2* (p*Sumo-2*) to pFLAG-*RARA* K399R (p*RARA*) cDNA constructs, treated with either vehicle or *at*RA (1 μM) for 30 min, fixed, and analyzed by immunofluorescence using anti-FLAG M2 antibody. Bar in a = 20 μM, for a-l. More RARA was found in the cytoplasm in SUMO-2 overexpressed cells after *at*RA treatment (arrow in f) compared to RARA with just endogenous SUMO-2 in cells treated with *at*RA (compare f with d or e).

Table 3.1: Primer sequences of SUMO specific protease (*Senp*) used for real-time RT-PCR

Gene	Forward (top) and reverse (bottom) primers (5'-…-3')
Senp1	ACTGCCATGTGTCTGCCTATGA CCCACTCCAGGACGGACTT
Senp2	GTTGAATGGGAGTGATTGTGGAAT CTGGTGCTGAGTGAATGTGATAGG
Senp3	GGTCCCTTGTCTCAGTTGATGTAAG GCGGTTTAGAGTTCGCTGTGA
Senp5	CGAGTGCGGAAGAGGATCTATAAG AGTCCCTGCTGAGTGAGTGTCA
Senp6	AACGGCACTGTAGCACTTACCA CTCGAAATGGGTCAGACACTTCT
Senp7	GTCTCAGCCCTCAAATGCAGAT GAGTAGCAGCCACTGCTTTGTTT
Senp8	TCTTTAGACGACAGCCAGAATCC ATTCTCCCCTCTTCTTTGTGATGTAT
S2	CTGCTCCTGTGCCCAAGAAG AAGGTGGCCTTGGCAAAGTT

CHAPTER 4

SUBCELLULAR LOCALIZATION OF SMALL UBIQUITIN-RELATED MODIFIER-2/3 (SUMO-2/3) IS MODULATED BY RETINOIC ACID IN THE TESTIS

This chapter was written as a manuscript for submission to Biology of Reproductive. I am the primary author, and the Fig. 4.7 was completed by Timothy J. Doyle.

Subcellular Localization of Small Ubiquitin-Related Modifier-2/3 (SUMO-2/3) is Modulated by Retinoic Acid in the Testis

Li Zhu, Timothy J. Doyle, and Kwan Hee Kim[1]

From School of Molecular Biosciences, Center of Reproductive Biology, Washington State University, Pullman, Washington 99164

Key Words: SUMO-2, Retinoic acid receptor alpha, retinoic acid, Sertoli cell, Testis

Running head: Characterization of SUMO-2/3 in Sertoli cells

[1] To whom correspondence should be addressed: Kwan Hee Kim, Ph.D., School of Molecular Biosciences, Washington State University, Pullman, WA 99164-4234. Tel: (509) 335-7022; Fax: (509) 335-1907; Email: khkim@wsu.edu.

4.1 ABSTRACT

Vitamin A is essential for spermatogenesis as demonstrated by the sterility of rat fed with vitamin A-deficient diet. The action of vitamin A is meditated by retinoid receptors. One of the retinoid receptors, retinoid acid receptor A (RARA) is critical for spermatogenesis, as shown by the mice lacking the *Rara* gene being sterile. The small ubiquitin-related modifer-2/3 (SUMO-2/3), are two highly related members of a family of ubiquitin-related proteins that form a polychain of SUMO-2/3 on the target protein. Our previous study has shown that SUMO-2 covalently modifies RARA and regulates the protein stability, cellular localization and transcriptional activity of RARA (unpublished data). However, the regulation of the expression and distribution of SUMO-2/3 *in vivo* remains unclear. Here, we report that *Sumo-2* and *Sumo-3* were widely expressed in various rat tissues, including testis. Having established that the SUMO-2/3 antibody primarily recognizes a large pool of free SUMO-2/3 on a Western blot, we used the antibody to determine the SUMO-2/3 expression during testis development. During early testicular development, SUMO-2/3 was mainly found in the nucleus of germ and Sertoli cells and shifted to the cytoplasm around day 15. Interestingly, the depletion of vitamin A in adult rat testes caused the translocation of SUMO-2/3 from the cytoplasm to the nucleus. Moreover, the disruption of *Rara* gene in adult mice also caused the similar localization change of SUMO-2/3, as that observed in VAD adult testes. Additionally, the depletion of vitamin A caused the decrease of free SUMO-2/3 and the increase of SUMO-2/3 conjugates in testes, but the disruption of *Rara* gene in mice did not change the protein expression of SUMO-2/3. In Sertoli cells, the cellular localization of SUMO-2/3 was changed upon *all-trans* retinoic acid (*at*RA) treatment, but not the protein expression. Hence, we conclude that *at*RA regulates the protein expression and cellular localization of SUMO-2/3 in testes through different signaling pathways.

4.2 INTRODUCTION

Vitamin A is crucial for spermatogenesis and overall health of human and animals [1-3]. When animals were fed on vitamin A deficient (VAD) diet, the testis size was decreased, as germ cells were depleted from the seminiferous tubules by apoptosis of early meiotic prophase spermatocytes and sloughing of haploid germ cells into the tubular lumen, exiting into the epididymis [4]. The only germ cells remaining in the VAD seminiferous tubules are preleptotene spermatocytes and type A1 spermatogonia [5]. Spermatogenesis could be reinitiated by a single injection of VAD animals with retinol [5, 6] and subsequent feeding of a vitamin A-sufficient diet. This retinoid replenishment essentially restores spermatogenesis, which can be synchronized to predictable, 3-4 spermatogenic stages of the seminiferous epithelial cycle in the regenerated testis [5-7].

Vitamin A signaling is mediated by retinoid receptors that include two types: the retinoic acid receptors (RAR) and the retinoid X receptors (RXR) [8]. Each has three major subtypes: alpha (A), beta (B) and gamma (G). These retinoid receptors belong to the superfamily of nuclear receptors that act as ligand-dependent transcription factors in the nucleus [8]. Basically, RARs heterodimerize with RXRs and bind to the retinoic acid response element (RARE) on target genes, modulating retinoic acid ligand-dependent transcription of the RARE-controlled genes [8]. All-*trans* retinoic acid (*at*RA) can activate both RAR and RXR, while 9-*cis* RA only activates RXR.

In the testis, all six retinoid receptors were expressed in either Sertoli cells and/or in germ cells [9]. Genetic ablation of *Rara* or *Rxrb* elicited abnormal testis phenotypes, observed as male sterility in affected animals [10-12]. The testicular morphology of the *Rara* knockout mice has been shown to be similar to that of VAD rats [10] and further characterization of *Rara* knockout mice revealed a role of RARA in synchronization of the spermatogenic cycle [13, 14]. In addition, RARA has been reported to have a potential to function in Sertoli cells to promote the survival and development of early meiotic prophase spermatocytes, whereas RARA in germ cells functions to increase the proliferation and differentiation of spermatogonia before meiotic prophase [15].

Recently, sumoylation, the covalent post-translational attachment of small ubiquitin-related modifier (SUMO) to target proteins, is attracting much attention. SUMO is ~ 100 amino acids and ~ 18% identical to ubiquitin [16]. There are four SUMOs, SUMO-1 to SUMO-4, in mammals and only three SUMO family members, SUMO-1 to SUMO-3, in human [16]. Mature SUMO-2 and SUMO-3 are ~95% identical to each other, but differ substantially from SUMO-1 [16]. SUMO-2/3 can form a poly SUMO-2/3 chain whereas SUMO-1 cannot. Mechanistically, sumoylation is a dynamic process, analogous to ubiquitylation. It involves a three-step enzymatic process in which SUMO is first activated by the SUMO E1-activating complex AOS1/UBA2 (SAE1/SAE2), before it is transferred to the SUMO E2-conjugating enzyme UBE2I (UBC9). Then, from the E2-conjugating enzyme, it is transferred to the substrate by three families of SUMO E3 ligases [17]: protein inhibitor of activated STAT (PIAS); Ran-binding protein 2 (Ranbp2) and polycomb protein 2(Pc2) [18]. Recently, it was shown that the SUMO polychain may be built and then transferred en bloc to the substrate and RanBP and the PIAS family may stimulate this multimerization process [19]. A set of highly active proteases can remove SUMO from target proteins [20]. A number of SUMO/sentrin specific isopeptidase (SENP) enzymes have been characterized and shown to have distinct subcellular localization patterns and non-redundant functions in the proteolytic processing of SUMO precursors, deconjugation of SUMO from the substrate, or editing of the SUMO 2/3 chain on the modified proteins [20] .

SUMO plays important roles in diverse reproductive functions, including gametogenesis, modulation of steroid receptor activity and ovulation [21-24]. During postembryonic development of *C. elegans*, the reproductive system is a major SUMO target, and SUMO is required for gonadal and uterine-vulvae morphogenesis [22]. SUMO-1 to 3 and UBC9 have been shown to be expressed in male germ cells during meiosis and in spermiogenesis [25]. SUMO-1 is postulated to have a role in sex chromosome silencing in spermatocytes, meiotic centromere function, and nuclear reshaping, potentially by sumoylating STAT-4 [26]. SUMO-2/3 is found to be correlated to abnormal spermatogenesis in human patients [27]. Moreover, SUMO-1 is found in Leydig and Sertoli cells in the testis [26]. In human Sertoli cells, interestingly, an inverse relationship between androgen receptor function and SUMO-1 expression

has been reported [28]. In our previous study, we found that the modification of RARA by SUMO-2 affected the subcellular localization, protein stability and transcriptional activity of RARA (unpublished data). However, little is known about the expression of SUMO-2/3 or the SUMO-related molecules in the testis, especially in Sertoli cells. Here, we used *in vivo* vitamin A deficiency and *Rara* knockout models, *in vitro* organ culture and Sertoli cell culture models, and various techniques to characterize the subcellular localization and protein expression of SUMO-2/3 in the testis, especially in Sertoli cells. We found that vitamin A signaling pathways regulate the distribution and expression of SUMO-2/3.

4.3 MATERIALS AND METHODS

Animals

Male Sprague-Dawley (SD) rats and male C57BL6 mice were obtained from an in-house vivarium. Animals, between 30–40 g body-weight, were placed on a VAD diet (Harland Teklad, Madison, WI) at 20 days of age. Testes were collected after 9 weeks on VAD diet. Animals were incapacitated with carbon monoxide anesthesia and sacrificed by cervical dislocation. To obtain retinol-replenished animals, the VAD animals were injected with 7.5 mg of all-*trans* retinol in 50% ethanol, followed by a dietary supplementation of 1 mg retinol/animal mixed with normal rodent diet (Harland Teklad, WI). Testes were collected after 4, 8, or 24 hrs post retinol injection. Institutional Animal Care and Use Committee of Washington State University approved the animal protocols that follow NIH guidelines.

Antibodies and Reagents

An affinity-purified rabbit polyclonal antibody raised against the full-length SUMO-2 protein was purchased from Zymed Laboratories (San Francisco, CA). A mouse monoclonal antibody raised against FLAG epitope and rabbit anti-actin antibody were purchased from Sigma-Aldrich, Inc (Saint Louis, MI). All-*trans* retinoic acid (*at*RA) was obtained from Sigma Aldrich (St Louis, MO). Am580 and Ro41-5253 was purchased from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA).

Primary Sertoli cells and Germ cells

Primary Sertoli cells were isolated from the testes of thirty 20-day-old rats by sequential enzymatic digestion as described in [9]. Decapsulated testis fragments were digested first with 0.25% (w/v) trypsin (Gibco BRL, Gaithersburg, MD) to remove the interstitial cells and then with collagenase (0.7 mg/ml, Sigma-Aldrich, St. Louis, MO). Sertoli cells were cultured in Ham's F-12 medium, maintained at 32 ^{o}C in a 5% CO_2 atmosphere up to 72 hrs. After culture medium was changed twice to reduce endogenous *at*RA, on the third day of culture, the cells were treated for 0, 2, 8, and 24 hrs with 1 μM of *at*RA (Sigma, St Louis, Mo).

Germ cells were isolated from the testes from two 70-day-old rats by sequential enzymatic digestion as described in [9]. Decapsulated testis fragments were digested first with collagenase (0.7 mg/ml) and then with 0.25% trypsin (w/v). The percentage of germ cells in each preparation was determined. The cells were smeared onto a slide, fixed with Bouin's solution for 1 hr, and stained with hematoxylin, and the number of germ cells was determined. More than 200 cells per preparation were counted. The average percentage of germ cells in three separate germ cells was 78.9 ± 0.6% (mean ± SD).

RNA Extraction and Semi-quantitative RT-PCR

Tissues from 20-day-old SD rats or cultured primary Sertoli cells, treated with either vehicle or *at*RA (1 μM), or Am580 (0.1 μM) for 24 hrs, were lysed using TRIzol reagent (Invitrogen, Calsbad, CA). The whole tissue samples were homogenized in a tissue homogenizer and the cultured Sertoli cells was passed through a pasture pipette to form a homogenous lysate in the presence of TRIzol reagent. Total RNA was isolated from the cell lysate and whole-tissue homogenate following the manufacturer's protocol. Total RNA (500 ng) was used to synthesize cDNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Each pair of primers, specific to the chosen genes, was designed using Primer express 2.0 software (Applied Biosystems, Foster City, CA) (Table 1). The cDNA was the template to conduct PCR, using specific primers for rat *Sumo-2 Sumo-3* and the control ribosomal RNA *S2*. Different amounts of

cDNA template were tested in order to reach the linear range. PCR products were resolved on 2% TAE-agarose gels, which was stained with ethidium bromide (0.4 mg/ml) for 10 min and then destained with H_2O for 10 min.

Protein Extraction and Western Blot Analysis

Soluble proteins from testes of adult rats, isolated germ cells and cultured Sertoli cells were collected as previously described [9]. Briefly, the testes or cells were homogenized in lysis buffer (50 mM Tris HCl [pH 8], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN). Cellular debris was pelleted by centrifugation at 12,000 x g for 15 min. The supernatant was separated by SDS-PAGE and subjected to electrophoresis, transferred to an Immobilon-P membrane (Millipore Co., Bedford, MA), blocked with 5% non-fat milk in Tris-buffered saline with Tween-20 (TBST) for 1 hr at room temperature and then incubated with primary antibody in TBST for 1 hr at room temperature, followed by incubation with horseradish peroxidase-conjugated antibody (Vector Laboratories, Burlingame, CA). Equal loading was determined using anti-actin antibody. Blots were developed with the ECL (enhanced chemiluminescence) Western blotting system (Amersham, Arlington Heights, IL) and exposed to X-ray film (Kodak, Rochester, NY).

Immunohistochemical Analysis

Testes from rats at age of 5, 10, 15, 20, 25, 30, 35 and 40 and 60 days were fixed in Bouin's for 4 hrs, embedded in paraffin, cut into 4μm thick sections, and mounted onto the slides coated with polylysine. The tissue sections were deparaffinized, rehydrated and microwaved in 50 mM Glycine (pH 3.5) for 15 min, cooled to room temperature and analyzed by immunohistochemical analysis as described previously [9]. Briefly, the tissues were blocked with 3% hydrogen peroxide, rinsed after 10 min in PBS, incubated with the SUMO-2/3 antibody solution (1:300) for overnight at 4 ^{o}C. Sections were rinsed with PBS, treated with biotinylated goat anti-rabbit secondary antibody (1:300, Vector Laboratories,

Burlingame, CA) for 1 hr at room temperature, followed by incubation with peroxidase-conjugated streptavidin and substrate-Chromagen mixture containing aminoethyl carbazole (Zymed Laboratories). As a negative control, serial sections were put through the same procedure without any primary antibody. All experiments were performed on the testicular sections from at least three different rats.

Experiments with Retinol in vivo

Intraperitoneal injections of retinol were in a volume of 100 μL, dissolved in ultrapure corn oil. Rats at age of 5 days were randomly divided into two groups. One group of rats was given 15 mg/kg retinol and the another group of rats was given equal volume of corn oil. After 4 hrs (P10 rats) or 5 days (P5 rats) injection, testes were taken and fixed in Bouin's for 4 hrs and analyzed as described in "Immunohistochemical Analysis".

Organ Culture

Testes from 10-day-old SD rats were obtained and cut into small pieces. One tissue from each animal was designated as a vehicle control, and its pair was subjected to RA treatment. The tissues were cultured in drops of media on Millicell-CM filters (Millipore, Bedford, MA) floating on the surface of 0.5 mL of CMRL Medium-1066 (Gibco, Grand Island, NY) supplemented with penicillin-streptomycin, insulin (10 μg/ml), transferring (10 μg/ml), L-glutamine (52 μg/ml), and BSA (0.1%) for 3 days and the medium was changed every 24 h. The tissues were collected after 72 hrs treatment, fixed in Bouin's for 4 hrs and analyzed as described in "Immunohistochemical Analysis".

Transient Transfection and Indirect Immunofluorescence

COS-7 cells were plated in a 24-well plate with cover slips and maintained with DMEM containing 10% FBS and penicillin (100 IU/mL), and streptomycin (100 μg/mL) at 37 ^{o}C in a saturated atmosphere of 5% CO_2. Two hours prior to transfection, the cells were maintained in DMEM containing

10% FBS without antibiotic and incubated under normal incubation conditions, and then transfected using Lipofectamine 2000 kit (Invitorgen).

Cultured primary Sertoli cells or transfected COS-7 cells were briefly washed with PBS, fixed with methanol at -20 ^{o}C for 15 min, washed with PBS, followed by incubation with either anti-SUMO-2/3 antibody (1:300) or anti-FLAG M2 antibody (1:500) overnight at 4 ^{o}C. After washed with PBS, cells were incubated with either goat anti-rabbit IgG or goat anti-mouse IgG (1:300 dilution) for 1 hr at RT, rinsed with PBS, followed by incubation with fluorescence avidin D (1:300) (Vector Laboratories) for 30 min at RT. Slides were mounted using Hard Set mounting media (Vector Laboratories). Digital images were obtained using a laser confocal system (Zeiss LSM 510, Hitachi, Japan) or a Leitz DMRB with epifluorescence and a Magnafire digital camera (Optronics, Goleta, CA).

RNA Extractions and Microarray Array Processing and Data Analysis

RNA was extracted from the 20 day-old cultured primary Sertoli cells treated with 1μM RA for 0, 2, 8, and 24hrs using an RNaqueous RNA extraction kit, following the manufacturer's protocol (Ambion, Austin, TX). Transcription profiling of RNA was performed using the Rat Expression 230 2.0 GeneChip® microarrays, that contain approximately 35,000 genes (Affymetrix, Santa Clara, CA), using one chip per RNA sample. Briefly, the double stranded cDNA, synthesized from 10μg total RNA, was used as template to prepare the antisense cRNA. 15μg biotin-labeled target cRNA was fragmented and hybridized with the GeneChip probe array, followed by washing steps, incubation with streptavidin phycoerythrin conjugate, and detection with Affymetrix Genechip® scanner 3000. Resulting image files were analyzed with Affymetrix GCOS software. The raw intensity data sets were normalized using default normalization parameters of Genespring 7.3 (Agilent Technologies, Foster City, CA). The normalized data for each sample were filtered for at least one sample having a raw signal of 50 or greater, and for at least one statistically significant change with a *p*-value of ≤0.05 for each gene, when comparing experimental time samples to each other. ANOVA tests were performed using a *p*-value of ≤0.05 for each gene, assuming variances not equal, and including calculations made from the Cross-Gene Error

Model. No multiple testing correction method was used and the Student-Newman-Keuls Post Hoc test was applied.

Statistical Analysis

Statistical analysis consisted of one-way ANOVA, followed by a pairwise comparison of the means at α=0.05 (Tukey-Kramer method, JMP; SAS institute Inc., Cary, NC).

4.3 RESULTS

SUMO-2 and SUMO-3 mRNA and protein were characterized in different rat tissues.

To determine the mRNA expression of SUMO-2/3 in rat tissues, semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using primers specific to rat *Sumo-2* and *Sumo-3*. *Sumo-2* was highly expressed in spleen, testis and pancreas and mildly expressed in heart and brain, but *Sumo-3* was highly expressed in prostate, testis, small intestine, heart, brain, skeletal muscle and kidney (Fig. 4.1A).

In order to determine whether SUMO-2/3 protein is expressed in the testis, proteins extracted from testes of rats at different ages, Sertoli cells isolated from P20 rats, and germ cells isolated from adult rats were subjected to Western blot analysis with anti-SUMO-2/3 antibody. The free SUMO-2/3 (15kDa) was predominantly present in the testis throughout the development to the adult (Fig. 4.1B) and in Sertoli and germ cells (Fig. 4.1C). Additionally, a few minor high molecular weight bands (37 and 50kDa), corresponding to SUMO-2/3 conjugates, were also observed in rat testes. Therefore, SUMO-2/3 mRNA and proteins were highly expressed in testes.

Cellular localization of SUMO-2/3 was characterized in developing and adult rat testes.

Since SUMO-2/3 antibody mainly recognized the free SUMO-2/3 (15 kDa) in Western blot analysis (Fig. 4.1B), we used the antibody to characterize the cellular localization of SUMO-2/3 in rat testicular sections from animals of different ages. Free SUMO-2/3 was strongly detected in the nucleus

of gonocytes and Sertoli cells (Fig. 4.2, A-D) in testes of P5 and P10 rats. Interestingly, SUMO-2/3 was observed in the cytoplasm of Sertoli cells and in the nucleus of early meiotic spermatocyte and spermatogonia in the testes of P15 rats (Fig. 4.2, E-F). SUMO-2/3 was also found in the cytoplasm of early meiotic and pachytene spermatocytes of P20 rats (Fig. 4.2, G and H), the nucleus of spermatogonia and the cytoplasm of pachytene spermatocytes and round spermatids of P25, P30 and P35 rats (Fig. 4.2, I-N). However, in adult rat testes, SUMO-2/3 was only found in the cytoplasm of pachytene spermatocytes and round spermatids, but not in elongated spermatids (Fig. 4.2, O-R). Taken together, SUMO-2/3 was consistently and predominantly present throughout the development and has different cellular localization patterns in juvenile, puberty rat testes and adult rat testes.

Disruption of vitamin A signaling impacted the subcellular localization of SUMO-2/3.

Given that the subcellular localization of SUMO-2/3 changed during testicular development, we determined whether *at*RA is one of the factors regulating the subcellular localization of SUMO-2/3. Immunohistochemical analysis was conducted in testicular sections from wild-type (WT), vitamin A-deficient (VAD) or retinol replenished VAD rats with anti-SUMO-2/3 antibody. When vitamin A signaling was disrupted in the testis, SUMO-2/3 was found in the nucleus of germ and Sertoli cells, while it was in the cytoplasm of cells in the WT testes (Fig. 4.3A-D), indicating that the disruption of vitamin A affected the subcellular localization of SUMO-2/3. With short-term retinol replenishment in VAD rat testes, the localization of SUMO-2/3 did not change, but with 96 hrs retinol replenishment, SUMO-2/3 was found in the cytoplasm of some cells in the tubules (Fig. 4.3E-L), suggesting that *at*RA regulated the subcellular localization of SUMO-2/3.

To investigate if exogenous *at*RA would affect the subcellular localization of SUMO-2/3 in young animals, 10-day-old rats were injected with retinol for 4 hrs or 5-day-old rats were injected with a single injection of retinol every 24 hrs for 5 days. In the testicular sections from rats injected with retinol for 4 hrs, no significant nuclear SUMO-2/3 staining was noticed (Fig. 4.3O-P), but strong nuclear SUMO-2/3 staining was observed in cells of the control testes (Fig. 4.3M-N). Moreover, in the testes injected

with retinol for 5 days, SUMO-2/3 staining was observed in the cytoplasm of cells (Fig. 4.3S-T), whereas it was found in the nucleus of cells in the control testes (Fig. 4.3Q-R), indicating that *in vivo* exogenous *at*RA affected the subcellular localization of SUMO-2/3 in the testes of young animals.

Furthermore, to determine if exogenous *at*RA would influence the subcellular localization of SUMO-2/3 *in vitro*, testes from 10-day-old rats were taken and cut into small pieces. Each pair of tissues was treated with either vehicle or *at*RA (1 μM) for 3 days. No significant nuclear SUMO-2/3 staining was observed in *at*RA-treated testes (Fig. 4.3V), but in the control testes, clear nuclear immunostain was seen in the nucleus of tesicular cells (Fig. 4.3U), indicating that *in vitro* exogenous *at*RA also affected the subcellular localization of SUMO-2/3 in the testes of young animals.

Disruption of RARA signaling pathway affected the cellular localization of SUMO-2/3.

Vitamin A signaling is mediated by retinoid receptor including RAR and RXR [8]. One of the retinoid receptors, RARA has been shown to be critical for spermatogenesis, as manifested by mice lacking the *Rara* gene being sterile [10]. To determine whether *at*RA affected the subcellular localization of SUMO-2/3 through the RARA signaling pathway, immunohistochemical analysis was performed on the testicular sections from *Rara* KO mice. SUMO-2/3 was found to be located in the nucleus of cells in *Rara* KO testes (Fig. 4.4C-D), similar to the pattern observed in adult VAD rat testes (Fig. 4.3C-D), but different from the pattern observed in WT testes (Fig. 4.4A-B), indicating that the disruption of RARA signaling pathway affects the subcellular localization of SUMO-2/3. Moreover, when *Rara* gene was disrupted in young animals, SUMO-2/3 was found in the cytoplasm in the testes of P7 (Fig. 4.4G-H) and P10 *Rara* KO mice (Fig. 4.4K-L), distinct from the localization pattern of SUMO-2/3 observed in the WT animals (Fig. 4.4E-F and 4.4I-J). Together, these results demonstrate that *at*RA through the RARA signaling pathway modulate the subcellular localization of SUMO-2/3 in the testis.

atRA through the RARA signaling regulated the subcellular localization of SUMO-2/3 in Sertoli cells

To evaluate if *at*RA modulate the subcellular localization of SUMO-2/3 in Sertoli cells, primary Sertoli cells were untreated or treated with *at*RA (1 μM) or a RARA specific agonist Am580 (0.1 μM) for 4 and 24 hrs. After treatment, cells were subjected to immunofluorescence analysis with anti-SUMO-2/3 antibody. In the absence of *at*RA, SUMO-2/3 was found in the nucleus of Sertoli cells (Fig. 4.5A-C), but in the presence of *at*RA SUMO-2/3 was observed in the cytoplasm of Sertoli cells with time, indicating that *at*RA regulated the translocation of SUMO-2/3 from the nucleus to the cytoplasm of Sertoli cells with time (Fig. 4.5D-I). Similar localization changes were observed in primary Sertoli cells treated with a RARA specific agonist Am580 (0.1 μM) (Fig. 4.5J-R), suggesting that *at*RA regulating the translocation of SUMO-2/3 in Sertoli cells is through the RARA signaling pathway.

Additionally, to eliminate the possibility of non-specific staining in Fig. 4.5A-R, COS-7 cells were transfected with FLAG tagged SUMO-2. After 24 hrs post-transfection, cells were untreated or treated with *at*RA (1 μM) for 4 and 24 hrs, and subjected to immunofluorescence analyses. In transfected COS-7 cells, the expressed SUMO-2 was observed in the nucleus without *at*RA, but in the presence of *at*RA, more SUMO-2 was found in the cytoplasm of Sertoli cells with time (Fig. 4.5S-U). Therefore, these results confirmed that *at*RA through the RARA signaling regulates the subcellular localization of SUMO-2/3 in Sertoli cells.

RA regulating the protein expression of SUMO-2/3 is not through RARA signaling in Sertoli cells

To determine whether *at*RA influenced the protein expression of SUMO-2/3 in primary Sertoli cells, Sertoli cell were untreated or treated with *at*RA (1 μM) for 2, 4, 8 and 24 hrs. Immunoblotting of cell lysates with anti-SUMO-2/3 antibody demonstrated that free SUMO-2/3 (15kDa) decreased with time (Fig. 4.6A and 4.6B), suggesting that the protein expression of free SUMO-2/3 in rat Sertoli cells was regulated by *at*RA. To determine whether the RARA signaling pathway affects the protein expression of SUMO-2/3 in primary Sertoli cells, the cells were untreated or treated with a RARA specific agonist Am580 (0.1 μM) or a RARA specific antagonist Ro-41-5253 (60 nM) for 2, 4, 6, and 24 hrs, respectively. The protein expression of free SUMO-2/3 was not changed by neither RARA specific agonist Am580 nor

RARA specific antagonist in primary Sertoli cells (Fig. 4.6D and E). Finally, free SUMO-2/3 was predominantly present in the testes of WT and *Rara* KO mice, and the disruption of RARA did not impact the expression pattern of SUMO-2/3 (Fig. 4.6E), indicating that *at*RA regulation was not through the RARA signaling pathway. Taken together, the protein expression of SUMO-2/3 in the testis is regulated by *at*RA, but the *at*RA-mediated regulation of SUMO-2/3 levels was not through the RARA signaling pathway.

A previous report showed that the cAMP-mediated subcellular distribution of SUMO-1 during decidualization may be due to the altered expression and regulation of E3 ligases and SENPs, which are expressed in distinct subcellular compartments [29]. Thus, we examined the expression of SUMO-related molecules in rat primary Sertoli cells by microarray analysis. The mRNA expressions of E1 activating enzyme *Uba2*, E2 conjugating enzyme *Ube2i* (*Ubc9*), and E3 ligases, *Ranbp2* and *Pias*1-4, as well as *Senp2, 3, 5, 6,* and *8*, and Nedd 8, the enzymes that antagonize E1,E2 and E3 enzymes (Fig. 4.7) were detected in Sertoli cells. Of these, *Uba2*, *Ube2i*, and *Pias2*, and *Senp6* were abundantly expressed with a signal of 1000 or more in Sertoli cells, indicating that they may be the E1, E2, E3, and SENP enzymes predominantly functioning in Sertoli cells. However, they were not regulated by *at*RA, except for *Pias1* that changed 3.7 fold after 2 hr *at*RA treatment of Sertoli cells.

4.5 DISCUSSION

SUMOs, a family of small ubiquitin-related proteins, and their associated proteins have been linked to a number of important cellular processes, including cell cycle regulation, nuclear-cytoplasmic trafficking, chromatin organization, oocyte growth, and spermatogenesis. SUMO-1, SUMO-2/3, and UBC9 have been reported to be present in male germ cells during meiosis and in spermiogenesis [25]. In human patient, abnormal level of SUMO-2/3, both low and high, has been shown to be correlated to azoospermia and hypospermatogenesis or reduction and impairment in the fidelity of spermatogenesis [27]. In this study, we analyzed the effect of *at*RA on the cellular and subcellular localization of SUMO-2/3 in the testis, especially in Sertoli cells. Previous studies have reported *Sumo-2* and *Sumo-3* mRNA

expression in the testis, specifically in germ cells, and colocalized SUMO-2/3 with UBE2I, the E2 enzyme for sumoylation, on the metaphase I chromosome of the spermatocytes [30].

By Western blots, we observed that the monomer SUMO-2/3 pool was predominantly present in the rat testis throughout the development to the adult stage (Fig. 4.1B and C). This is consistent with a previous study that reported a large pool of free or non-conjugated SUMO-2/3, which appeared to be readily available for conjugation reactions induced by cellular stresses such as acute heat elevation and oxidative stress [31]. Alternative explanations for having a large pool of SUMO-2/3 is that SUMO proteases such as SENP6, also known as SUSP1, which can specifically dismantle SUMO-2/3 chains, are highly expressed in the testis [32].

Interestingly, immunohistochemical analysis showed that SUMO-2/3 was translocated from the nucleus to the cytoplasm of testicular cells after day 15 in rats. During the normal testis development, germ cells usually start to enter into pre-meiotic DNA synthesis and meiosis around day 10 to 15 in rats. Curiously, previous studies have shown that SUMO-2/3 is present in human meiotic spermatocytes as early as leptotene and zygotene, but not SUMO-1 [27], and SUMO-2/3 and UBE2I (UBC9) are detected near centromeres in metaphase I spermatocytes [25]. These studies indicate that SUMO-2/3 is an important element for meiotic progression in the testis. Interestingly, leptotene and zygotene are the same cells that have SUMO-2/3 in the nucleus in the testes of P15 rats in this report (Fig. 4.2, E-F).

The different SUMO-2/3 subcellular localization patterns observed in the early and late testis development suggest some factors may be regulating the SUMO-2/3 subcellular localization. We investigated whether vitamin A could be one of the factors affecting the subcellular localization of SUMO-2/3 and examined the SUMO-2/3 subcellular localization in rat testes during vitamin A depletion and, retinol and *at*RA additions. During vitamin A depletion, the testis undergoes degeneration by either germ cell death or loss of germ cells by sloughing [33] and only contains preleptotene spermatocytes, type A1 spermatogonia and Sertoli cells [34]. In VAD rat testes, SUMO-2/3 was immuno-stained in the nucleus of the remaining germ cells and Sertoli cells, whereas it was found in the cytoplasm of germ cells and Sertoli cells in normal adult rat testes (Fig. 4.3A-J). However, with a long term retinol replenishment

in VAD rat testes, the cytoplasmic SUMO-2/3 staining was observed in some tubules, but not with a short term retinol replenishment (Fig. 4.3K-L). In addition, the similar subcellular localization changes were observed in the retinol-injected testes and in organ-cultured testes treated with *at*RA (Fig. 4.3M-V). These results suggest that the subcellular localization of SUMO-2/3 in the testes is regulated by *at*RA.

The *at*RA effects are mediated by a RARA-mediated mechanism. The disruption of *Rara* gene in adult and young animals caused the similar subcellular localization changes of SUMO-2/3, as observed in organ cultures (Fig. 4.4). Moreover, SUMO-2/3 was found to translocate from the nucleus to the cytoplasm of isolated primary Sertoli cells upon treatment with Am580, a RARA-specific agonist (Fig. 4.5J-R). Although the underlying molecular mechanism requires future investigation, these results clearly suggest that the *at*RA-regulated subcellular localization of SUMO-2/3 is through the RARA signaling pathway.

Additionally, the *at*RA decreased the Sertoli cell expression of SUMO-2/3 by Western blot analysis (Fig. 4.6B). Previously, the androgen 5α-dihydrotestosterone decreased SUMO-2/3 in prostrate [35] as well as the LH receptor stimulated inhibition of the SUMO-1 expression during ovulation in mouse granulose cells [24]. However, neither a RARA specific agonist Am580 nor a RARA specific antagonist Ro-41-5253 significantly affected the expression of free SUMO-2/3 (Fig. 4.6C-D), indicating that the Sertoli cell expression of SUMO-2/3 was regulated by *at*RA, but not through a RARA signaling pathway. Similarly, neither the free SUMO-2/3 nor the high molecular weight SUMO-2/3 conjugates was influenced by the disruption of *Rara* gene in mice (Fig. 4.6E).

Sumoylation of a substrate protein in cells reflects the steady-state equilibrium of sumoylation and desumoylation processes. Some of the desumoylating enzymes contain multiple nuclear localization domains and are thought to dictate the subcellular distribution of SUMO conjugates [36]. Regulation of SENPs, PIAS and Pc2 E3 ligases during decidualization appear to control the nuclear targeting of SUMO-1 [29]. Thus, it is interesting that E1 activating enzyme *Uba2*, E2 conjugating enzyme *Ube2i* (*Ubc9*), and E3 ligases, *Ranbp2* and *Pias*1-4, as well as families of *Senps* and *Nedds* were detected in Sertoli cells, some of them, such as *Uba2*, *Ube2i*, and *Pias2*, and *Senp6* were highly expressed in Sertoli

cells (Fig. 4.7). SENPs are known to discriminate between processing, deconjugating, and editing substrates, process the SUMO-1 to 3 with different affinities, and has a preference for subcellular localizations [37]. UBE2I has been shown to be colocalized with SUMO-1 and SUMO-2/3 to the XY body in pachytene and diplotene spermatocytes, while SUMO-2/3 and UBE2I were detected near centromeres in metaphase I spermatocytes in male germ cells [25]. In oocytes, the UBE2I did not completely colocalize with either SUMO-1 or SUMO-2/3, but UBE2I was found in nuclear speckles, the location associated with mRNA processing and active transcription [38]. Moreover, SENP6 (SUSP1) with multiple potential nuclear localization domains is located in the cytoplasm and the nucleus [37] and is known to preferentially edit a SUMO-2/3 polychain [39-42]. SUMO-2/3 accumulated in PML nuclear bodies in cells that were treated with SENP6 RNAi [32]. In addition, RanBP2 auto-stimulates polychain formations [18] These all point to SUMO-2/3, sumoylation enzymes and SENPs, especially SENP6, together having a critical role in Sertoli cells and *at*RA potentially having control over the SUMO-2/3 function by increasing sumoylation, perhaps by increasing the expression of Pias1. Further investigation is required to determine the exact role that each conjugating enzyme and protease plays in the testis and Sertoli cells. Nonetheless, given that hypoSUMO-2/3 and hyperSUMO-2/3 cause problems in spermatogenesis in human [27], it could be a promising strategy to manipulate SUMO-2/3 function by *at*RA to treat such patients.

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Table 4.1. Primer sequences of Sumo-2 and Sumo-3 used for semi-quantitative RT-PCR

Gene	Forward (top) and reverse (bottom) primers (5'-…-3')
Sumo-2	GGCAACCAATCAACGAAACAG TGCTGGAACACATCAATCGTATC
Sumo-3	ACCCAGCCATTTTCCTGACAT GCGGACTCTTGTGTGATTGGT
S2	CTGCTCCTGTGCCCAAGAAG AAGGTGGCCTTGGCAAAGTT

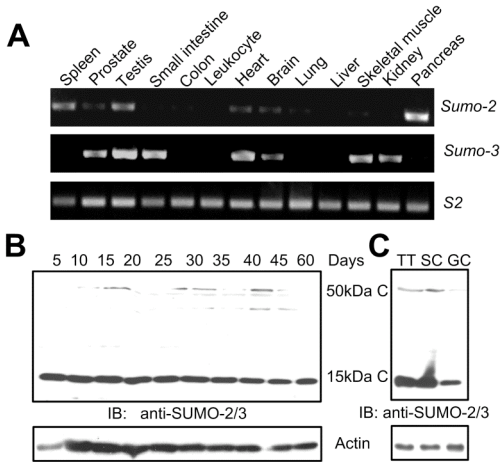

FIGURE 4.1. **SUMO-2 and SUMO-3 mRNA and protein were characterized in the testis.** A. RNA from different tissues isolated from 20-day-old SD rats was obtained and analyzed for *Sumo-2* and *Sumo-3* by semi-quantitative RT-PCR. Equal loading was determined by *S2*. B-C. Proteins isolated from rats at different ages and testicular cells were subjected to immunoblotting (IB) with anti-SUMO-2/3 antibody or anti-actin antibody. Equal loading was determined by actin. TT, total testis; SC, Sertoli cells; GC, germ cells. All experiments were repeated at least three times.

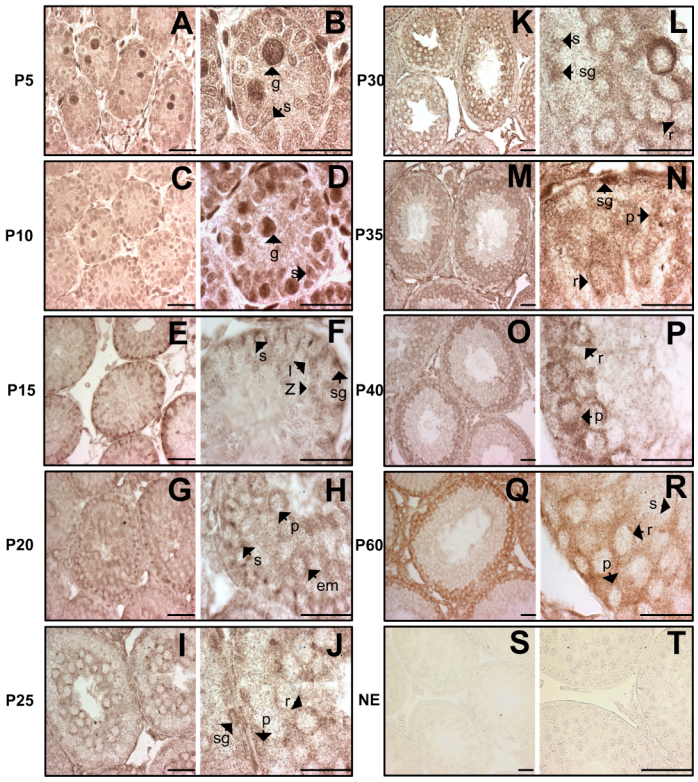

FIGURE 4.2. **Cellular localization of SUMO-2/3 was characterized in developing and adult rat testes.** Immunohistochemical analysis with anti-SUMO-2/3 antibody was performed on testicular sections from rats at age of 5 (A-B), 10 (C-D), 15 (E-F), 20 (G-H), 25(I-J), 30 (K-L), 35 (M-N), 40 (O-P) and 60 (Q-R) days. A testicular section from a 30-day-old rat was incubated with anti-SUMO-2/3 antibody only as a negative control (NE) (S-T). g, gonocyte; sg, spermatogonia; s, Sertoli cells; l, leptotene; z, zygotene; p, pachytene spermatocyte; r, round spermatid. Bar in A = 50 μm for A, C, E, G and I; bar in B = 20 μm for B, D, F, H, J, L, N, P, R and T; bar in K = 100 μm for K, M, O, Q, S. Photographs were representative of at least four experiments.

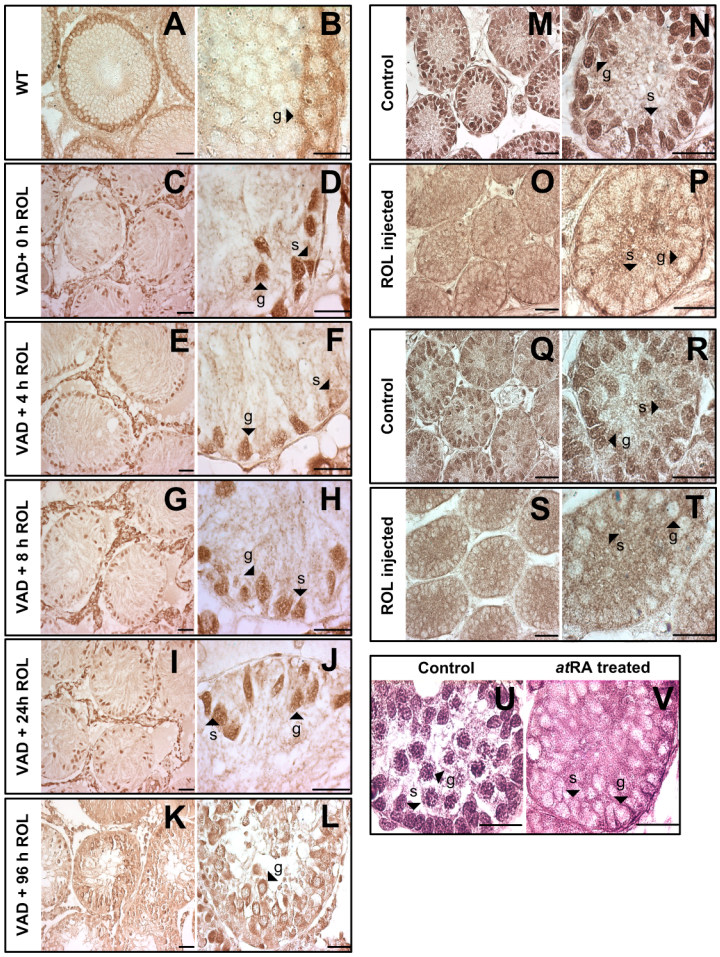

FIGURE 4.3. **Disruption of vitamin A signaling influenced the protein expression and subcellular localization of SUMO-2/3 in the testis.** Immunohistochemical analysis with anti-SUMO-2/3 antibody was performed on testicular sections from adult WT rats, VAD rats, and VAD rats replenished with retinol for each indicated times (A-L), or from 10-day-old rats injected with retinol (ROL, 15 mg/kg) for 4 hrs (M-P), or from 5-day-old rats injected with retinol once every 24 hrs for 5 days (Q-T), or from *in vitro* cultured testicular sections of 10-day-old rats (U-V). g, germ cell; s, Sertoli cell; WT, wild-type; VAD, vitamin A deficient. Bar in A = 20 μm for A, C, E, G, I, K; bar in L = 50 μm for L, M, O, Q, S; bar in B = 100 μm for B, D, F, H, J, N, P, R, T. Photographs were representative of at least four experiments.

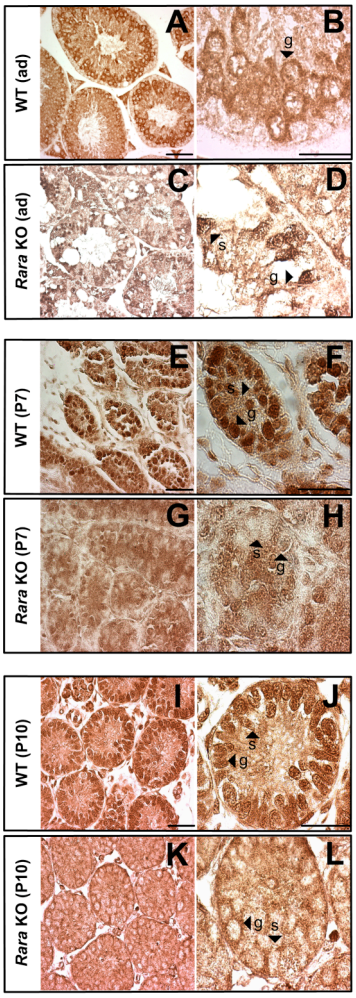

FIGURE 4.4. **Disruption of RARA signaling affected the subcellular localization of SUMO-2/3 in the testis.** Immunohistochemical analysis with anti-SUMO-2/3 antibody was performed on testicular sections from adult wild type (WT) and adult *Rara* knockout (KO) mice (A-D), or from 7-day-old WT and *Rara* KO mice (E-H), or from 10-day-old WT and *Rara* KO mice (I-L). g, germ cell; s, Sertoli cell. Bar in A = 20 μm for A, C; bar in E = 50 μm for E, G, I, K; bar in B = 100 μm for B, D, F, H, J, L. Photographs were representative of at least three experiments.

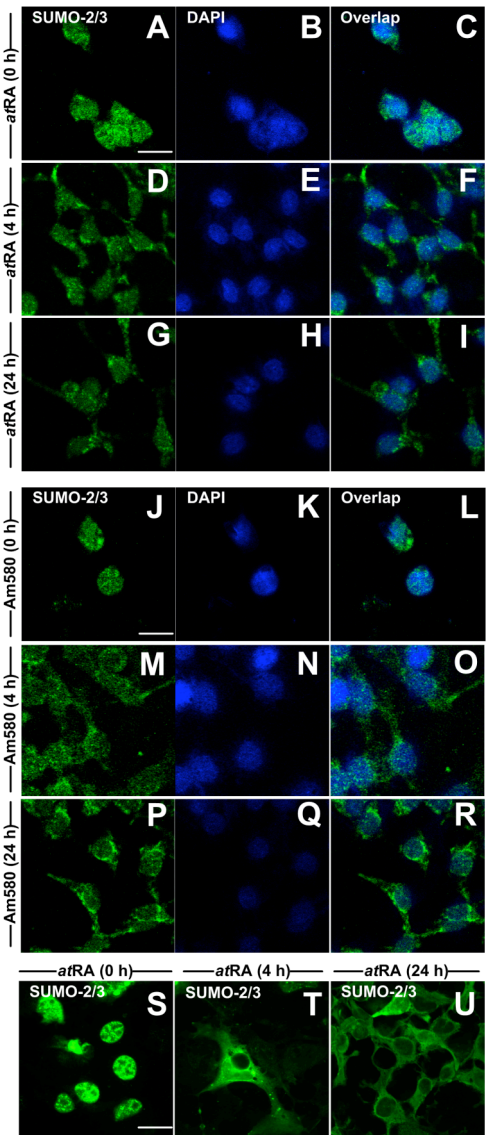

FIGURE 4.5. ***at*RA through the RARA signaling regulate the subcellular localization of SUMO-2/3 in Sertoli cells.** Primary Sertoli cells were treated with *at*RA (1 μM) (A-I) or Am580 (0.1 μM) (J-R) for 0, 4 and 24 hrs, and subjected to indirect immunofluorescence analysis with anti-SUMO-2/3 (left panels) antibody. DAPI stained nucleus in blue color. Merged images in right panels. COS-7 cells, transfected with pFLAG-*Sumo-2* cDNA construct, were treated after 24 hrs with *at*RA (1 μM) for 0, 4 and 24 hrs, and then subjected to indirect immunofluorescence analysis with anti-FLAG M2 antibody (S-U). Photomicrographs were representative of at least three experiments.

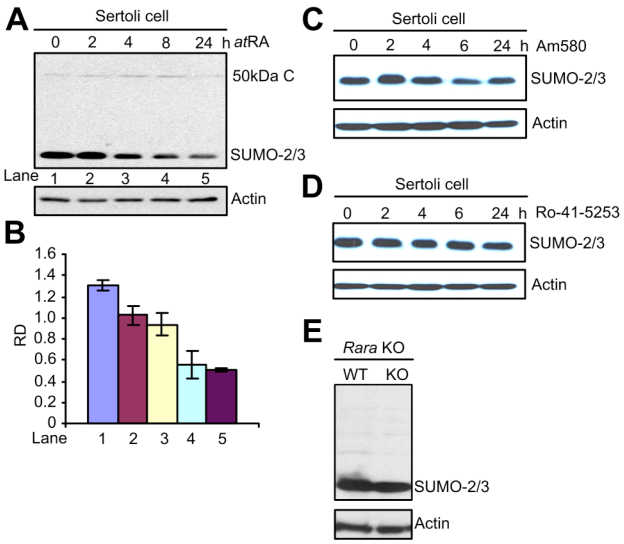

FIGURE 4.6. *at*RA **regulating the protein expression of SUMO-2/3 is not through RARA signaling in Sertoli cells.** Proteins, isolated from cultured primary Sertoli cells treated with *at*RA (1 μM) (A), or Am580 (0.1 μM) (C), or Ro-41-5253 (60 nM) (D) for indicated time, or from adult WT or *Rara* knockout (KO) mice (E), were subjected to immunoblotting with anti-SUMO-2/3 or anti-actin antibody. B. The intensities of monomer SUMO-2/3 (A) were scanned and graphed as means ± SEM from n=3 independent experiments. RD, relative density. Equal loading was determined by anti-actin antibody. All experiments were repeated at least three times.

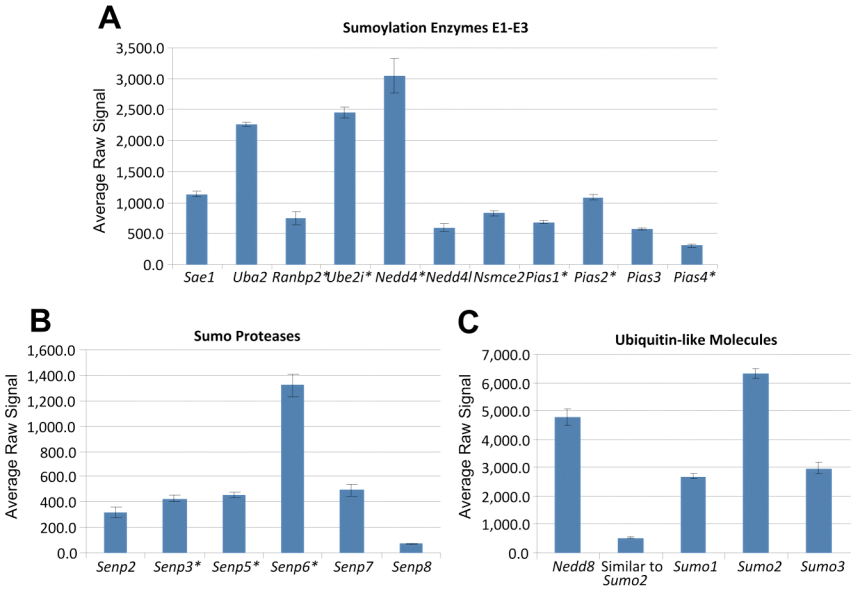

FIGURE 4.7. **Relative levels of mRNA for SUMO related genes in cultured primary Sertoli cells.** Relative levels of mRNA for SUMO E1-E3 enzymes (A) or SUMO specific proteases (B) or ubiquitin-like molecule (C) in cultured primary Sertoli cells. *, signals from multiple probes were averaged. Values represent relative level of each gene ± SEM from three replicates.

CHAPTER 5

GLUCOSE-REGULATED PROTEIN 58 (GRP58) DELIVERS RETINOIC ACID RECEPTOR ALPHA (RARA) TO THE NUCLEUS AND THEN TO THE ER FOR DEGRADATION IN SERTOLI CELLS

This chapter was written as a manuscript to be submitted to *Journal of Biological Chemistry*. I am the primary author and performed all the experiments.

Glucose-regulated Protein 58 (GRp58) Delivers Retinoic Acid Receptor Alpha (RARA) to the Nucleus and then to the ER for Degradation in Sertoli Cells

Running Title: Interaction between RARA and GRp58

Li Zhu and Kwan Hee Kim[1]

[1]School of Molecular Biosciences, Center for Reproductive Biology, Washington State University, Pullman, Washington 99164

Correspondence to: Kwan Hee Kim, Ph.D. School of Molecular Biosciences, Washington State University, Pullman, WA 99164-4234. Tel: (509) 335-7022. Fax: (509) 335-1907. Email: khkim@wsu.edu

Key Words: GRp58, ERp57, PDIA3, 1,25D3-MARRS receptor, Retinoic acid receptor alpha, RARA, nuclear localization, chaperone, oxidoreductase, Sertoli cells

5.1 ABSTRACT

Binding of the ligand, all-trans retinoic acid (*at*RA), to retinoic acid receptor alpha (RARA) triggers the receptor translocation from the cytoplasm to the nucleus, where it activates the transcription of target genes, critical for many physiological processes. Here, we demonstrate a novel finding that the nuclear-cytoplasmic trafficking of RARA is regulated by glucose-regulated protein 58 (GRp58) that is also known as a disulfide isomerase (PDIA3) or $1,25D_3$-MARRS (membrane-associated, rapid response steroid binding) receptor that binds vitamin D metabolites, $1,25(OH)_2D_3$. GRp58 mRNA and protein were highly expressed in germ cells and Sertoli cells, and in the testis throughout animal development. GRp58 interacted with RARA and, upon *at*RA treatment, the complex was translocated from the cytoplasm to the nucleus and, then with time, GRp58 dissociated from RARA and was exported to the cytoplasm. The GRp58 RNAi treatment disrupted *at*RA-dependent RARA nuclear localization, indicating that GRp58 is required for the nuclear localization of RARA. Moreover, the treatment with sulfhydryl modifying agents that modify cysteine residues abolished *at*RA-mediated RARA nuclear localization, suggesting that the thiol-oxidoreductase activity of GRp58 are required for the nuclear localization of RARA. Additionally, the proteasome inhibitor treatment resulted in the retention of GRp58 and RARA close to the endoplasmic reticulum (ER), suggesting that GRp58 may accompany RARA to ER for the ER associated degradation (ERAD), before it de-couples from RARA. Collectively, the results indicate that GRp58 is a molecular chaperone that utilizes sulfhydryl groups to deliver RARA to the nucleus and, then with time, to the ER for degradation in Sertoli cells.

5.2 INTRODUCTION

Retinoic acid is a potent trigger of many physiological processes that include growth, differentiation of somatic and germ cells, and meiosis in germ cells. Its action is mediated by the ligand-dependent transcriptional factors, retinoid receptors, which belong to the superfamily of nuclear steroid/thyroid hormone receptors. The ligand binding induces a conformation change that favor co-activator tethering to the receptors, switching on the transcription of genes containing retinoic acid response element (RARE) (1). This assembled protein complex at the regulated promoters induces chromatin remodeling and increases the affinity of RNA polymerase II to these promoters, thereby stabilizing the transcriptional initiation complex and the transcriptional activation of target genes (1). Before moving into the nucleus, nuclear receptors, such as glucocorticoid receptor (2, 3), progesterone receptor (4, 5), estrogen receptor (6, 7), form a multiprotein heterocomplex that contains heat shock protein 90 (Hsp90), Hsp70, and an immunophilin, such as FKBP51 or Cyp40. Hsp70 and Hsp90 are molecular chaperones for maintaining an appropriate ligand-binding conformation for nuclear receptors, and are known to participate in the nuclear-cytoplasmic shuttling and the decay of nuclear receptors (8-10). For instance, it has been proposed that, in the presence of its ligand, glucocorticoid receptor alpha (GRA) moves from the cytoplasm to the nucleus in GRA-Hsp90-immunophilin complex form, in which immunophilin acts as a protein transport unit of the transportosome (11, 12). Similarly, retinoid receptors, RAR and RXR, have been shown to interact with Hsp90, which is required for transcriptional activity of RAR or RXR-RAR heterodimer from two types of retinoic acid response elements, triggered by three different ligand agonists (13). Hence, it is clear that molecular chaperone proteins are a group of the key molecules that may be required to ensure proper functions of nuclear receptors, including RARs.

Genetic studies have shown that one of the retinoid receptors, retinoic acid receptor alpha (RARA) is critical for spermatogenesis, as shown by a sterility phenotype observed in *Rara* knockout mice (14). Further characterization has revealed that RARA plays an important role in the synchronization of the spermatogenic cycle (15, 16). In *Rara* knockout mice, a normal proportion of the spermatogenic stages

of the seminiferous epithelial cycle could not be maintained, instead, a major disorganization of germ cell association was observed (15, 16). Meanwhile, it has been reported that RARA might function in the somatic nurse cells, Sertoli cells, to promote the survival and development of early meiotic prophase spermatocytes, whereas in germ cells, RARA might function to enhance the proliferation and differentiation of spermatogonia before meiotic prophase (17). Thus, unraveling the regulatory processes by which the RARA transcriptional activity change in the germ cells and Sertoli cells is a significant task. To better understand the molecular elements regulating the RARA transcriptional activity in Sertoli cells, we performed a yeast two-hybrid screening using RARA as the bait and the protein products of a primary Sertoli cell cDNA expression library as the prey. One of the proteins that strongly interacted with RARA was Glucose-related protein 58 (GRp58).

GRp58, also known as ERp57 and PDIA3, is a thiol-oxidoreductase and an isoform of protein disulfide isomerase (PDI). It is composed of an endoplasmic reticulum (ER) recognition signal sequence at the N-terminal end, two thioredoxin-like domains and a nuclear localization signal in the middle, and an ER retention motif QDEL at the C-terminal end (18). The redox activity of the thioredoxin-like domains is provided by two cysteine residues present in a characteristic CXXC sequence (18). It is reported that one-third of GRp58 can be cytoplasmic, whereas the majority is in the lumen of ER (19), as well as in the nuclear matrix (20). GRp58 has an extensive role in protein maturation, post-translational modification, and protein folding processes which have been associated with many diseases such as Alzheimer's, prion neurotoxicity, cancer, and hepatotoxicity (21). Particularly, GRp58, similar to other protein disulfide isomerases (PDI), isomerizes both native and non-native disulfide bonds, catalyzed by the thiol oxidoreductase activity, thus facilitating the eventual acquisition of the native conformation of a protein (22).

In the ER, GRp58 is known to function as a subunit of specific complexes formed with the resident ER chaperone proteins, calreticulin and calnexin that require Ca^{2+} for their activities (23). Calreticulin and calnexin are molecular lectins that recognize a glycoprotein with a single terminal glucose on the N-linked oligosaccharide as an incompletely trimmed intermediate — an incompletely

folded glycoprotein — and help to fold the glycoprotein correctly (23). If the glycoprotein has attained its native conformation, the glycoprotein no longer has a single glucose left on the N-linked oligosaccharide, thus is not recognized by calnexin or calreticulin, and then it exits ER to Golgi (24). If not, the substrate is either subjected to additional folding cycles or selected for degradation by ER-associated degradation (ERAD) machinery. ERAD involves retrotranslocation of misfolded proteins to the cytoplasm, where they are deglycosyled, ubiquitinated by ER-bound ubiquitin-conjugating enzymes such as the Ubc6e E2 enzyme and E3 RMA1 ubiquitin ligase, and degraded by proteasomes (24, 25). Thus, a misfolded variant of α_1-antitrypsin with its glycans in the $Glc_1Man_8GlcNAc_2$ form (Glc, glucose; Man, mannose; $GlcNAc_2$, N-acetyl glucosamine) is retained in the ER and stably associated with calnexin, upon proteasome inhibitor treatment (26).

In addition, GRp58 is also known as the $1,25D_3$-MARRS receptor, a membrane-associated rapid response receptor that binds vitamin D active metabolite, $1,25(OH)_2D_3$. It is able to translocate to the nucleus of intestinal cells upon $1,25(OH)_2D_3$ binding, where it may interact with transcription factors and/or DNA (21) and activates protein kinase C (PKC) activity, important for the regulation of calcium channels in the kidney or intestinal cells (21). This effect does not involve the classical vitamin D receptor (21). Moreover, GRp58 has been demonstrated to be important in the testis. It is found to undergo post-translational modification during sperm capacitation (27). Blocking GRp58 with antibodies significantly inhibits human sperm from penetrating zona-free hamster oocytes in a dose-dependent manner (27). Different inhibitors of protein disulfide isomerase (PDI) activity were able to inhibit sperm-egg fusion *in vitro* (28). However, little is known regarding the protein substrates of GRp58 or the mechanism by which it can regulate its substrate in the testis. Here, we evaluated the role of GRp58 in regulating the transport of RARA and demonstrated that GRp58 might be a novel molecular chaperone for the nuclear import and subsequent proteolytic processing of RARA at the ER.

5.3 EXPERIMENTAL PROCEDURES

Antibodies and Reagents — Goat anti-GRp58 antibody, goat anti-calreticulin and rabbit anti-RARA antibody were purchased from Santa Cruz biotechnology, Inc. (Santa Cruz, CA). FITC-conjugated anti-rabbit antibody, anti-FLAG M2 and anti-Histidine (HIS) monoclonal antibodies were obtained from Sigma-Aldrich, Inc (Saint Louis, MI). Texas red-coupled anti-goat antibody was purchased from Vector Laboratories, Inc. (Burlingame, CA). Sulfhydryl modification agents, methyl methanethiol sulfonate (MMTS) and 5, 5'-Dithiobio(2-nitro-benzoic acid) (DTNB), were purchased from Sigma-Aldrich, Inc.

Plasmid Constructs — A h*RARA* cDNA from LRARaSN (29) was subcloned into pFLAG-CMV2 vector (Sigma-Aldrich Co., St Louis, MO), pBD-GAL4 (Stratagene, La Jolla, CA) and pQE-Tri-system (Qiagen, Inc., Valencia, CA). Total RNA, isolated from 20-day-old rat Sertoli cells using TRIzol reagent (Invitrogen, Calsbad, CA), was used to generate cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The synthesized cDNA was used to conduct PCR to generate GRp58 cDNA using specific primers (5'-TCGCGGCCGCATGCCGCTTCAGCTGCCTTGC-3' and 5'-TCATCGATTTAGAGGTC CTCTTGTGCCTT-3'). The amplicon was digested with NotI and ClaI and subcloned into pFLAG-CMV2 vector (Sigma-Aldrich).

Yeast Two-Hybrid Screening — Yeast strain AH109 (Clontech Laboratories, Inc., Mountain View, CA) containing pBD-GAL-h*RARA* was mated with Y187 (Clontech Laboratories) with the rat Sertoli cells cDNA expression library in pAD-GAL4 and plated on medium lacking histidine (HIS), adenosine-2 (ADE2), leucine (LEU) and tryptophan (TRP) and supplemented with 3-amino-triazole (Sigma-Aldrich) to decreased histidine background. HIS^+, $ADE2^+$, LEU^+, TRP^+ colonies were measured for β-galactosidase activity using the filter lift assay (30). HIS^+, $ADE2^+$, LEU^+, TRP^+ colonies exhibiting high β-galactosidase activity (Lac Z+ colonies) were further characterized. To recover cDNA library plasmids, total DNA from HIS^+, $ADE2^+$, LEU^+, TRP^+, $LacZ^+$ colonies was isolated and used to transform *Escherichia coli* DH5α (Invitrogen). To ensure that the correct cDNAs were identified, cDNA library

plasmids isolated were transformed into AH109 containing pBD-GAL-h*RARA* and plated on medium lacking histidine, adenosine-2, leucine, and tryptophan and supplemented with 3-amino-triazole. *β*-galactosidase activity was determined from HIS^+, $ADE2^+$, LEU^+, TRP^+ colonies using the filter lift assay.

Cell Culture, Transfection and RNA interference — COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), supplemented with penicillin (100 IU/mL) and streptomycin (100 μg/mL) at 37 ^oC in a saturated atmosphere of 5% CO_2. Primary Sertoli cells were isolated from the testes of 20-day-old rats by sequential enzymatic digestions (31). Decapsulated testis fragments were digested first with 0.25% (w/v) trypsin (Gibco BRL, Gaithersburg, MD) to remove the interstitial cells, and then with collagenase (0.7 mg/mL, Sigma-Aldrich), and maintained in Ham's F-12 medium (Invitrogen Laboratories, Carlsbad, CA) in a 32 ^oC 5% CO_2 atmosphere. Animal protocols, following the NIH guidelines, were approved by the Institutional Animal Care and Use Committee of Washington State University.

COS-7 cells or primary Sertoli cells, grown in 24-well plates or 60 mm plates, were transiently transfected with 6 μg/60 mm plate or 1 μg/well (24-well plate) of a 1:1 ratio of GRp58 and RARA cDNA expression constructs or 20 μM GRp58 siRNA or control siRNA using Lipofectamine 2000 reagent (Invitrogen). After 48 hr post-transfection, cells were either untreated or treated with each indicated reagents, and harvested for pull-down assay, immunoblotting, or immunofluorescence.

Semi-quantitative RT-PCR — Total RNA, isolated from different tissues of 20-day old SD rats using TRIzol reagent, was used to synthesize cDNA using iScript cDNA Synthesis Kit (Bio-Rad). The generated cDNAs were substrates to conduct PCR with primers specific for rat GRp58 (5'-TCGCGGCCGCATGGGCTTCAGCTGCCTTGC-3') and (5'-TGATCGATTTAGAGGTCCTCTTGTG CTT-3'), and ribosomal protein S2 (5'-CTGCTCCTGTGCCCAAGAAG-3') and (5'-AAGGTG GCCTTGGCAAAGTT-3'). For the quantitation of GRp58, S2 was used as a loading control. The number

of PCR cycles was determined by performing a series of RT-PCR reactions with different PCR cycles to make sure PCR amplification was in the log linear phase.

Western blotting — Western blot analyses were performed as described previously (32). Proteins were subjected to SDS-PAGE and transferred to Immobilon-P membranes (Millipore Co., Bedford, MA). After blocking with 5% non-fat milk in tris-buffered saline with 0.5% Tween (0.5% TBST) for 1 hr, membranes were incubated with primary antibody and then with horseradish peroxidase-conjugated secondary antibody both in 0.5% TBST for 1 hr at room temperature (RT). Equal loading was determined using Western blot analysis with anti-actin antibody. Blots were processed with Enhanced Chemiluminescence Western blotting system (Amersham Biosciences, Piscataway, NJ).

Immunohistochemistry — Testes, taken from rats at age of 5, 10, 15, 20, 25, 30, 35, 40 and 60 days, were fixed in Bouin's for 4 hrs, embedded in paraffin, cut into 4μm-thick sections, and mounted onto the slides coated with polylysine. The tissue sections were deparaffinized, rehydrated, and microwaved in 50 mM glycine (pH 3.5) for 15 min, cooled to RT, and rinsed in 3% hydrogen peroxide for 10 min. Then, tissue sections blocked with 10% normal rabbit serum (Vector Laboratories) in PBS for 30 min at RT, incubated with anti-GRp58 antibody (1:100), followed by incubation with the biotinylated rabbit anti-goat secondary antibody (1:300) (Vector Laboratories). As a negative control, serial sections were put through the same procedure without primary antibody.

His and FLAG pull-down assay — For His pull-down assay, cells were lysed by adding ice-cold lysis buffer (50 mM HEPES-KOH (pH 7.5), 200 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 10% (v/v) Triton X-100), supplemented with a protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN). After centrifugation, a part of the supernatant was taken as the input. The remaining supernatant was diluted to 1 ml using the same cold lysis buffer containing protease inhibitors, mixed with 50 μL of Ni-NTA magnetic beads (Qiagen Inc., Valencia, CA) and incubated at 4 ^{o}C on an end-over-end shaker for

overnight. After incubation, the tubes were placed on a magnet for 1 min and the supernatant removed from the separated beads using a pipette. The separated beads were washed with 1.5 mL cold lysis buffer three times, followed by 1.5 mL of the same buffer with 20 mM imidazole and 1.5 mL TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Between each wash, the tubes were placed on a magnet for 1 min. After final wash, SDS-PAGE loading buffer was added to elute the complex, analyzed by Western blot.

For FLAG pull-down assay, cells were lysed by adding lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100), supplemented with a protease inhibitor cocktail tablet. After centrifugation, a part of the supernatant was used as the input. The remaining was mixed with 40 µL of anti-FLAG M2 affinity gel (Sigma-Aldrich), pre-cleaned by mixing with the lysate, and followed by incubation on an end-over-end shaker for overnight at 4 ^{o}C. The resin was centrifuged for 30 seconds at 5,000 g and the supernatants were removed. Then, the resin was washed with TEand mixed with SDS-PAGE loading buffer to elute the complex for Western blot analysis.

Indirect immunofluorescence and confocal microscropy — Primary Sertoli cells, seeded in 24-well plates, were fixed with -20 ^{o}C methanol for 10 min, blocked with 10% normal goat serum (Vector Laboratories, Inc.) for 1 hr, before incubation with anti-RARA and anti-GRp58 or anti-calreticulin antibodies overnight at 4 ^{o}C. After wash, the RARA antibody was visualized by a FITC-conjugated secondary antibody (Sigma-Aldrich), whereas GRp58 and calreticulin were detected with Texas red-coupled secondary antibody (Vector Laboratories). All digital images were obtained using a laser confocal system (Zeiss LSM 510, Hitachi, Japan).

5.4 RESULTS

GRp58 interacted with RARA in yeast two-hybrid screening

To investigate RARA interacting proteins, a yeast two-hybrid screen was conducted. Full-length RARA was cloned into a GAL4 DNA binding domain yeast expression vector (pGAL4-DBD RARA), and this was used as the bait to screen a rat Sertoli cells cDNA library. The cDNA library from 20-day-

old rat Sertoli cells was constructed and introduced as a translational fusion with the GAL4 transactivating domain [GAL(AD)-cDNA] into Y187 yeast strains as previously described (33). RARA-interacting clones were identified by their ability to activate reporter constructs with the UAS_{GAL4} when cotransformed with GAL(DBD)-RARA. Of a particular interest, we isolated a ~1.7 kb cDNA clone that was sequenced and compared to the genbank database using the BLAST search program. This clone contained an open reading frame of 1515 bp (505 amino acids) and showed 100% identity to rat GRp58 (NM_017319) (Fig. 5.1A). GRp58 is a 58kDa protein and has a N-terminal ER recognition signal, two thioredoxin active sites, a nuclear localization signal next to a ER retention signal QEDL sequence at the C-terminal (Fig. 5.1A).

RARA interacted with GRp58

To confirm the interaction between RARA and GRp58 in mammalian cells, COS-7 cells, which have a low detectable endogenous RARA, were cotransfected with either pHis empty vector or pHis-h*RARA* and pFLAG-*GRp58* cDNA expression constructs. Proteins, interacting with HIS-RARA, were captured using nickel-nitrilotriacetic acid (Ni-NTA) magnetic agarose beads and identified by immunoblotting with anti-FLAG M2 antibody. GRp58 co-purified with HIS-RARA in the extracts from the cells transfected with pHis-GRp58, but not in the extracts from the cells bearing the empty vector, indicating that GRp58 was associated with RARA in COS-7 cells (Fig. 5.1B). Furthermore, in reverse, proteins interacting with FLAG-GRp58 were captured using anti-FLAG M2 affinity gel and identified by immunoblotting with anti-HIS antibody. RARA was co-purified with FLAG-GRp58 from the extracts of the cells transfected with pFLAG-GRp58 cDNA expression construct, but not from the extracts of the cells bearing the empty vector (Fig. 5.1C), suggesting again that RARA interacted with GRp58 in COS-7 cells.

GRp58 mRNA and protein were highly present in rat testes

To determine the location of GRp58 mRNA expression in male rats, total RNA, extracted from a panel of different tissues from 20-day-old rats, was used in semi-quantitative RT-PCR. Of all rat tissues examined, GRp58 mRNA was highly expressed in prostate and testis, and weakly present in small intestine and heart (Fig. 5.2A). Additionally, we examined GRp58 mRNA expression in the testis and found that it was highly expressed in both Sertoli cells and germ cells (Fig. 5.2B). Moreover, to determine the GRp58 protein expression in the testis, proteins were extracted from adult rat testes, isolated germ cells from adult rat testes and Sertoli cells from 20-day-old rats and analyzed by Western blot using anti-GRp58 antibody. The GRp58 protein was also highly present in germ cells and Sertoli cells (Fig. 5.2C). Therefore, both GRp58 mRNA and protein were highly expressed in germ cells and Sertoli cells in rat testes.

Immunohistochemical analysis of GRp58 expression in developing and adult rat testes

To characterize the cellular localization of GRp58 in testes, immunohistochemical analysis was performed on testicular sections collected from adult rats or rats at age of 5, 10, 15, 20, 25, 30, 35 and 60 days during the first wave of spermatogenesis. GRp58 was present throughout the development to the adult stage in rat testes (Fig. 5.2D-S). In the early development, GRp58 was mainly localized on the membrane and/or in the cytoplasm of Sertoli cells (Fig. 5.2, D-M). Similarly, in germ cells, the subcellular localization of GRp58 was primarily on the membrane and/or the cytoplasm of Sertoli cells, although some germ cells expressed GRp58 in the nucleus at postnatal day 10 (P10). Around P30, GRp58 was found specifically in the acrosomal structure of round spermatids (Fig. 5.2, N-O). Afterwards, GRp58 was observed in the nucleus of the early pre-meiotic germ cells, the same cells that express a high level of RARA (34) and the elongated spermatids, as well as in the acrosomal structure of round spermatids (Fig. 5.2, P-S).

RARA co-localized with GRp58 in the presence or absence of *at*RA

To determine whether RARA co-localizes with GRp58 in primary Sertoli cells, time-dependent immunofluorescence studies were conducted using anti-RARA and anti-GRp58 antibodies. Both RARA and GRp58 were found to co-localize at the periphery of the nucleus and in the cytoplasm of Sertoli cells (Fig. 5.3A-C). This is consistent with previous results that demonstrated that RARA is primarily located in the cytoplasm of Sertoli cells in the absence of ligand (35) and GRp58 is partially in the endoplasmic reticulum and partially in the cytoplasm [20]. To determine whether GRp58 co-localizes with RARA after *at*RA treatment, primary Sertoli cells were treated with *at*RA (1 μM) for 2, 4 or 8 hrs, fixed and immunostained with anti-RARA and anti-GRp58 antibodies. Upon 2 hrs *at*RA treatment, RARA was primarily in the nucleus (Fig. 5.3D), as previously reported (35). As indicated by the overlap (Fig. 5.3F), some GRp58 was in the nucleus, co-localized with RARA, although an appreciable amount of GRp58 was also in the cytoplasm (Fig. 5.3E and F). Interestingly, upon 4 hrs *at*RA treatment, RARA was near the periphery of the nucleus, while GRp58 was mainly in the cytoplasm (Fig. 5.3G-I), indicating that RARA and GRp58 may have dissociated from each other by 4 hrs. After 8 hrs of *at*RA treatment, the majority of GRp58 was found in the cytoplasm (Fig. 5.3K and L), while RARA was still located near the periphery of the nucleus (Fig. 5.3J and L), although the amount of RARA is a lot less at 8 hrs.

To determine whether RARA, located to the periphery of the nucleus, is on or in the endoplasmic reticulum (ER), primary Sertoli cells were treated with *at*RA for 2 or 4 hrs, fixed and immunostained with anti-RARA and anti-calreticulin antibodies. Calreticulin is a soluble ER resident protein, often used as an ER marker (36), and is shown to be associated with GRp58 in the ER (23). Within 2 hrs *at*RA treatment, although some of RARA were overlapped with calreticulin in the ER, RARA was primarily translocated into the nucleus (Fig. 5.3M and 3O). More interestingly, upon 4 hrs *at*RA treatment, the majority of RARA were co-localized with calreticulin in the ER (Fig. 5.3P-R), indicating that RARA was located on or in the ER within 4 hrs. Since *at*RA seems to increase the intensity of GRp58 immuno-staining with time, we conducted Western blot analysis on cell extracts isolated from Sertoli cells treated with *at*RA for

2, 4, and 8 hrs. Indeed, it showed that the amount of GRp58 in Sertoli cells increased with *at*RA treatment (Fig. 5.3S).

Disruption of GRp58 by RNAi interfered with the nuclear localization of RARA

To determine the role of GRp58 in the nuclear localization of RARA, we disrupted the expression of endogenous GRp58 using RNAi technology in primary Sertoli cells. Primary Sertoli cells, transfected with GRp58 specific siRNA or control siRNA, were collected after 48 hrs and lysed for RNA or protein extraction. GRp58 specific siRNA was able to efficiently knockdown more than 70% of the endogenous GRp58 RNA (Fig. 5.4A) and protein (Fig. 5.4B). Then, primary Sertoli cells, transfected with either control siRNA or siRNA specific to GRp58, were treated with either vehicle or *at*RA (1 μM) for 2 hrs, fixed and immunostained with anti-RARA and anti-GRp58 antibodies. Strikingly, in the cells transfected with GRp58 specific siRNA, only strong RARA staining was observed in the cytoplasm in the absence or presence of *at*RA (Fig. 5.4C, E, F, and H), indicating that the disruption of endogenous GRp58 interfered with the nuclear localization of RARA. In the cells transfected with control siRNA, both RARA and GRp58 were consistently overlapped in the cytoplasm of Sertoli cells in the absence of *at*RA (Fig. 5.4I-K) and, upon 2 hrs *at*RA treatment, they were co-localized in the nucleus of Sertoli cells (Fig. 5.4L-N). Taken together, the data suggest that GRp58 is required for the nuclear localization of RARA in Sertoli cells

Disruption of *at*RA binding to RARA by sulfhydryl modifying agents influenced the nuclear localization of RARA

To determine whether GRp58 works as a disulfide isomerase with the thiol-oxidoreductase activity, sulfhydryl modifying agents, MMTS and DTNB, were used to block thiol-disulfide interchange reaction of the disulfide isomerase. Previously, these sulfhydryl modifying agents were shown to modify cysteine residues in the ligand-binding domain of RARA and block *at*RA binding to RARA (37, 38). Primary Sertoli cells were pre-treated with MMTS (1 mM) for 30 min and then treated with *at*RA and

MMTS, or treated with MMTS alone for 2 hrs, fixed and immunostained with anti-RARA and anti-GRp58 antibodies. Interestingly, in the presence of *at*RA and MMTS, both RARA and GRp58 were found in the cytoplasm of Sertoli cells (Fig. 5.4O-Q), which was opposite to the localization pattern observed in the presence of *at*RA (Fig. 5.3D-F). This indicates that *at*RA binding to RARA was required for the nuclear co-localization of RARA and GRp58. As expected, RARA and GRp58 were in the cytoplasm of Sertoli cells in the presence of MMTS alone (Fig. 5.3R-T). Additionally, experiments with a lower concentration of MMTS (0.5 mM) (Fig. 5.4U-W) and another sulfhydryl modifying reagent DTNB (100 μM) (Fig. 5.4X-Z) showed the similar results. Taken together, the nuclear localization of RARA requires the efficient *at*RA binding to RARA, which probably relies on functional cysteine residues in the ligand binding domain. Thus, the thiol oxidoreductase activity of GRp58 could be involved in the *at*RA binding to RARA.

MG132 changed the subcellular localization of RARA and GRp58 at 4 hrs *at*RA treatment

It is known that *at*RA triggers RARA to undergo degradation by the ubiquitin-mediated proteasome pathway immediately after the transcription of its target gene (39). In fact, it is known that RARA still attached to the response elements of the target gene is doubly kinased and subsequently degraded through ubiquitin-mediated proteasome pathway (39, 40). To determine if the ubiquitin-mediated proteasome pathway has a role in the dissociation of RARA with GRp58 at 4 hrs after *at*RA treatment, primary Sertoli cells were either pre-treated 30 min with MG132 (10 nM), a reversible inhibitor of proteasome, and then treated with *at*RA and MG132, or MG132 alone for 4 hrs, fixed and immunostained with anti-RARA and anti-GRp58 or anti-calreticulin antibodies. Calreticulin and RARA were both observed co-localized on or in the ER of Sertoli cells in the presence of *at*RA and MG132 (Fig. 5.5A-C), indicating that the inhibition of the proteasome-mediated degradation did not change the RARA location close to the ER. Similarly, GRp58 was found mainly on or in the ER, overlapping with RARA (Fig. 5.5D-F), different from predominantly cytoplasmic localization of GRp58 in the absence of MG132 (Fig. 5.3H and I). This strongly suggests that the inhibition of the proteasome-mediated degradation

abolished the export of GRp58 from the nucleus to the cytoplasm. On the other hand, the cells were treated with MG132 alone for 4 hrs (Fig. 5.5G-I), both RARA and GRp58 were observed in the cytoplasm. Thus, MG132 alone did not affect the co-localization pattern of RARA and GRp58. Together, these results suggest that GRp58 may accompany RARA to the ER for RARA degradation, and when RARA is degraded, GRp58 is recycled back to the cytoplasm.

MG132 disrupted the dissociation of RARA and GRp58 at 4 hrs *at*RA treatment

To determine whether *at*RA affects the interaction between RARA and GRp58 with time, COS-7 cells were treated with either vehicle or *at*RA for 4 hrs, after transfection with pHis-h*RARA* cDNA and pFLAG-*GRp58* cDNA expression constructs. Additionally, COS-7 cells were transfected with increasing amounts of pFLAG-*GRp58* to make sure that *GRp58* was not limiting. Proteins, interacting with HIS-RARA, were captured using Ni-NTA magnetic agarose beads. The amount of GRp58 co-purified with HIS-RARA upon 4 hrs *at*RA treatment was significantly less compared to that of GRp58 in untreated sample (Fig. 5.6A and 6B), indicating that *at*RA in 4 hrs induced the dissociation of GRp58 and RARA. The decrease in GRp58 with time is not due to the amount of GRp58 present in the cell, since *at*RA was actually shown to increase the steady state level of GRp58 (Fig. 5.3S). Furthermore, the similar transfection experiments were performed with the pretreatment of MG132 (10 nM), a reversible inhibitor of proteasomes, and 2 hrs or 4 hrs *at*RA treatment. Interestingly, upon MG132 treatment, the amount of GRp58 co-purified with HIS-RARA was significantly higher compared to that of GRp58 in 4 hrs *at*RA treated sample (compared Fig. 5.6C and Fig. 5.6B), and similar to that of GRp58 in 2 hrs *at*RA treated sample (Fig. 5.6C), indicating that MG132 blocks the *at*RA-induced dissociation of RARA and GRp58.

5.5 DISCUSSION

Molecular chaperones such as Hsp90 have been highlighted to play a critical role in modulating the biological response of nuclear receptors (3). Hsp90 is an established drug target, and its inhibitor geldanamycin, effectively blocked ligand-binding ability of progesterone receptor, glucocorticoid

receptor and mineralocoriticoid receptor, and also promoted the degradation of nuclear receptors (41-43). The ligand binding and transcriptional activation of RAR was also impaired in a yeast lacking Hsp90 (13). These data suggest that molecular chaperones are required for the normal functions of nuclear receptors. Recently, endoplasmic reticulum chaperones, such as GRp58 (ERp57), have been reported to be responsible for protein folding as well as other functions such as binding vitamin D active metabolite, $1,25(OH)_2D_3$, the regulation of calcium homeostasis, and activation of specific transcription factors (44).

Here, we report that GRp58 was strongly associated with RARA in Sertoli cells using a full-length RARA as the bait and protein products expressed from a Sertoli cell cDNA library as the prey in a yeast two-hybrid screening. This interaction was confirmed in transfected COS-7 cells (Fig. 5.1B and C and Fig. 5.6), indicating that RARA and GRp58 interacted in mammalian cells. GRp58 mRNA was highly expressed in prostate and testis, but weakly expressed in small intestine and heart of all the examined rat tissues (Fig. 5.2A-C). In the early testis development, GRp58 was located in the membrane and/or the cytoplasm of germ cells and Sertoli cells (Fig. 5.2D-M). It was mainly found in the acrosome of the round spermatids starting around 30 days (Fig. 5.2N-O) and continued its expression in developing spermatids, which is consistent with previous studies (28, 45). Additionally, we report a novel finding that GRp58 is in the nucleus of pre-meiotic germ cells close to the basement membrane (Fig. 5.2R-S).

In Sertoli cells, we found that both RARA and GRp58 were translocated from the cytoplasm to the nucleus and overlapped in the nucleus (Fig. 5.3D-F). This is similar to previous findings that GRp58, as a membrane receptor of $1,25(OH)_2D_3$, is mobilized from the cytoplasm to the nucleus upon $1,25(OH)_2D_3$ treatment in IEC-6 cells (48) and glucocorticoid receptor alpha (GRA) moves from the cytoplasm to the nucleus as a GRA-Hsp90-immunophilin complex form (11, 12). Interestingly, when GRp58 was knocked down by RNAi technique, RARA was observed in the cytoplasm of Sertoli cells, even in the presence of *at*RA (Fig. 5.4F and H), indicating that GRp58 is required for the nuclear localization of RARA. We also observed that the nuclear co-localization of RARA and GRp58 was disrupted by pretreatment with sulfhydryl modifying agents MMTS (Fig. 5.4O-P) and DTNB (Fig. 5.4X-Z), which modify the cysteine residues. Previous study has shown that cysteines in the ligand-binding

domain of RARA are involved in ligand-receptor interactions and the ligand-binding activity of RARA is markedly decreased by sulfhydryl modifying agents such as MMTS and DTNB (37, 38). Thus, taken together, it appears that cysteine residues are important for the *at*RA-dependent nuclear localization of RARA, and the thiol oxidoreductase activity of GRp58 may be required for the thiol exchange reactions with the cysteine residues of RARA for enhancing the *at*RA binding to RARA. Future experiments are needed to verify the exact molecular mechanism.

Curiously, the interaction of GRp58 with RARA and the subcellular localization patterns of GRp58 and RARA changed with time. In the absence of *at*RA, GRp58 with RARA were co-localized in the cytoplasm (Fig. 5.3A-C). With *at*RA treatment, GRp58 with RARA were found co-localized to the nucleus in 2 hrs (Fig. 5.3D-F), but the majority of RARA were located in the ER (Fig. 5.3G, I, J, and L), while GRp58 was localized to the cytoplasm in 4 and 8 hrs (Fig. 5.3H, I, K, and L). More interestingly, in the presence of MG132 and *at*RA, RARA, GRp58, and calreticulin were co-localized in the ER (Fig. 5.5A-F), suggesting that the inhibition of proteasome-mediated degradation pathway caused the retention of GRp58 and RARA in the ER. This implies that before GRp58 was able to translocate back to the cytoplasm, it had to accompany RARA to the ER. This is consistent with the decreased level of GRp58 associating with HIS-RARA in the cells treated with *at*RA at 4 hrs, significantly less compared to that in the untreated cells (Fig. 5.6A). Moreover, the amount of GRp58 copurified with HIS-RARA was significantly higher in the presence of both *at*RA and MG132, a reversible inhibitor of proteasomes, in 4 hrs than the amount of GRp58 in *at*RA alone treated samples (Fig. 5.6B and C). These results suggest that *at*RA induced the degradation of RARA, as expected, but also led to disassociation of GRp58 from RARA. Based on these findings, our speculation is that upon *at*RA treatment, GRp58 accompanys RARA to the ERAD machinery, the cell's protein quality control system. Whether calreticulin or calnexin serve as a conduit for RARA degradation is not known, although it was reported previously that calreticulin physically interacts with RARA, as shown by co-immunoprecipitation (36). More intriguingly, a corepressor, N-CoR, associated with PML-RARA, a fusion protein of promyelocutic leukemia (PML) and RARA that causes acute promyelocytic leukemia (APL), was shown undergo the ERAD, including

ubiquitylation of N-CoR via an ubiquitin-conjugating enzyme, Ubc6 (46). This report suggests that a reduction in N-CoR protein levels in the nucleus could be partially responsible for uncontrolled growth in APL.

In conclusion, we have identified GRp58 as a molecular chaperone for RARA and it participates in *at*RA binding and the nuclear localization of RARA. RNAi and proteasome inhibitor studies have elucidated that GRp58 is essential for the nuclear localization of RARA and it accompanies RARA to the ER. Sulfhydryl modifying agent studies allow us to speculate that GRp58 may work as a thio oxidoreductase to enhance the ligand binding to RARA. Hence, we propose a model (Fig. 5.7) that RARA interacts with GRp58 to form a RARA-GRp58 complex in the cytoplasm of Sertoli cells. Upon *at*RA binding, both RARA and GRp58 are translocated into the nucleus. During the process, GRp58 may work as a thio oxidoreductase to enhance the *at*RA binding to RARA. Within the nucleus, the RARA-GRp58 complex binds to the retinoic acid response element and initiates the transcription of target genes. Afterwards, GRp58 accompanies the used RARA to the ERAD machinery and then recycles back into the cytoplasm for the next cycle. Thus, we demonstrate that manipulation of GRp58 could be an alternative mechanism to modulate the functions of RARA in Sertoli cells.

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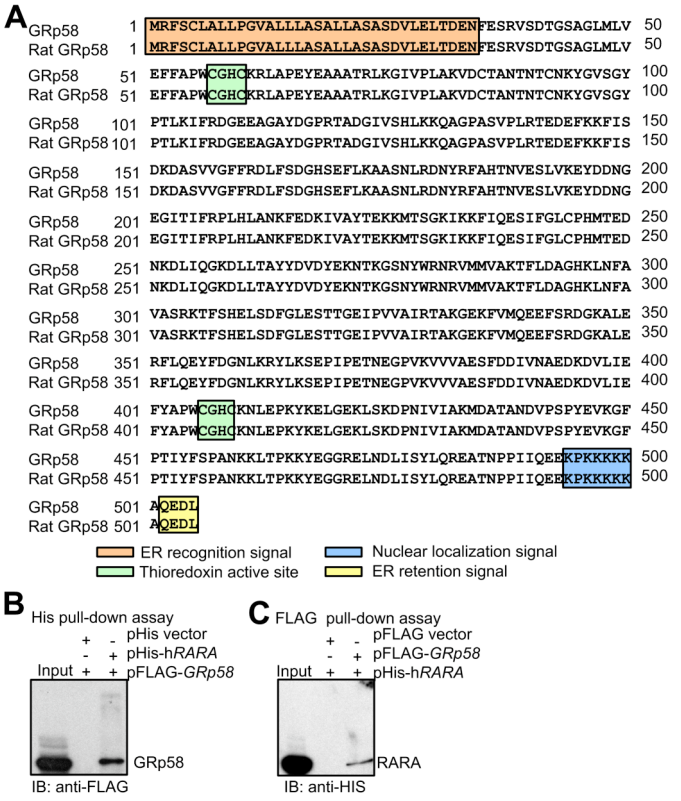

FIGURE 5.1. **GRp58 interacted with RARA.** A. The translated protein sequence of *GRp58* cDNA isolated from a LacZ-positive yeast clone, identified as containing a protein that strongly interacts with RARA. The GRp58 sequence was identical to rat GRp58 (NM_017319). Different motifs are boxed. B-C. COS-7 cells, transfected with either empty vector and/or pHis-h*RARA* and pFLAG-*GRp58* cDNA expression plasmids, were harvested after 48 hrs and subjected to either His pull down assay (B) or FLAG pull-down assay (C) followed by immunoblotting with anti-FLAG M2 (B) or anti-HIS antibody (C). The input, the same lysate used for pull-down assay. All experiments were repeated three times.

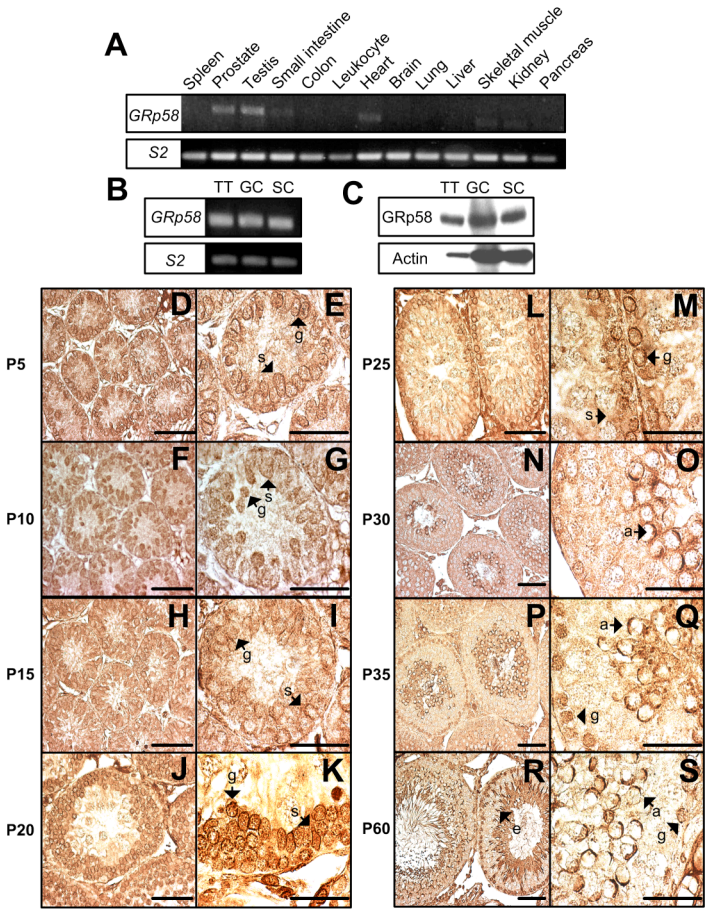

FIGURE 5.2. **GRp58 mRNA and protein are expressed in rat testes.** A-B. Total RNA was isolated from each indicated tissue and analyzed by semi-quantitative RT-PCR with primers specific for rat GRp58. Equal loading was determined by S2. C. Proteins extracted from the testis tissue from 20-day-old rats, cultured primary Sertoli cells and isolated germ cells were subjected to immunoblotting with anti-GRp58 or anti-actin antibody. Equal loading was determined by anti-actin antibody. D-S. Immunohistochemical analysis was conducted using anti-GRp58 on testicular sections from rats at age of 5 (D-E), 10 (F-G), 15 (H-I), 20 (J-K), 25 (L-M), 30 (N-O), 35 (P-Q), and 60 (R-S) days. Bar in D= 50 μm for D, F, H, J and L; bar in E = 100 μm for E, G, I, K, M, O, Q and S; bar in N = 20 μm for N, P and R. s, Sertoli cell; g, germ cell; a, acrosome; e, elongated spermatid. All experiments were repeated three times.

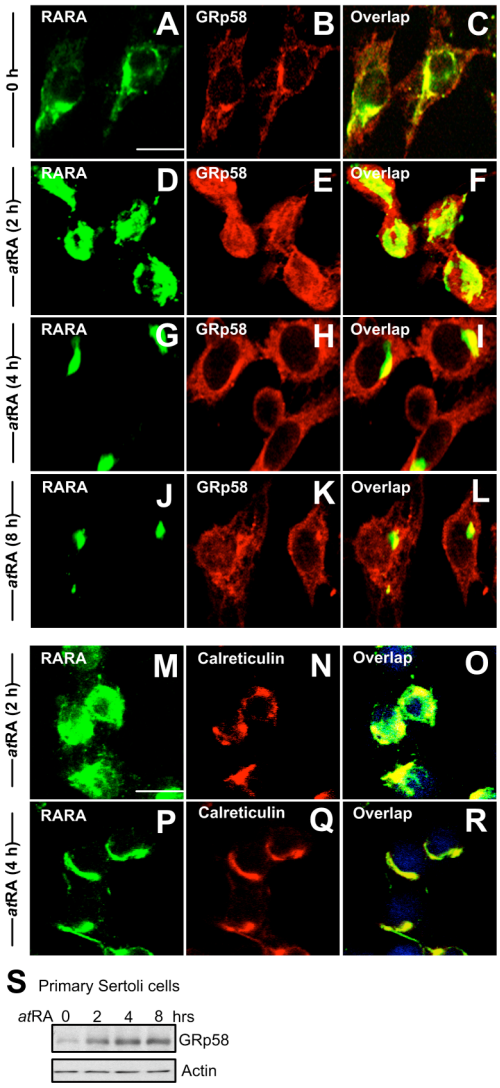

FIGURE 5.3. **GRp58 co-localized with RARA in the presence or absence of *at*RA.** A-R. Primary Sertoli cells were treated with either vehicle or *at*RA for 2, 4 or 8 hrs, fixed and subjected to immunofluorescence analysis with anti-RARA (A, D, G, J, M, and P) and anti-GRp58 (B, E, H, and K) or anti-calreticulin (N and Q) antibodies. Merged images in yellow color in C, F, I, L, O, and R. Bar in A and M = 100 μm for A-R. S. Primary Sertoli cells were treated with either vehicle or *at*RA (1 μM) for 2, 4 or 8 hrs, lysed and subjected to immunoblotting with anti-GRp58 or anti-actin antibody. Equal loading was determined by anti-actin antibody. All experiments were repeated at least three times.

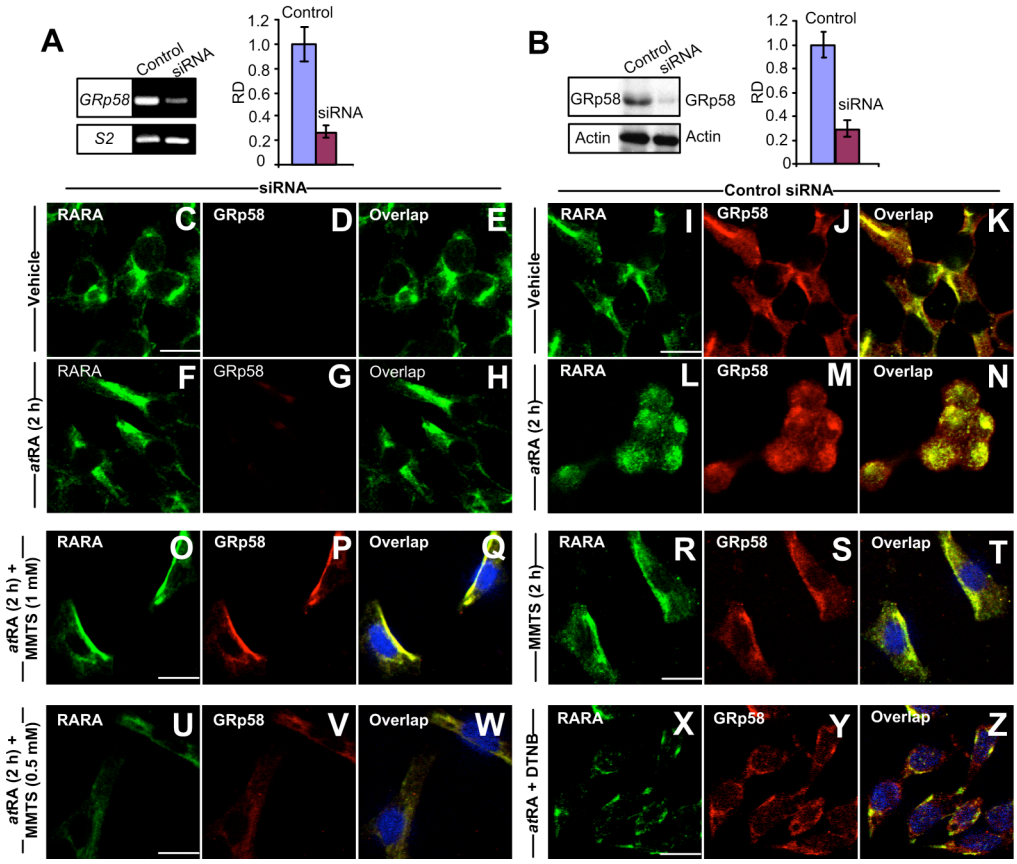

FIGURE 5.4. **GRp58 is required for the nuclear localization of RARA**. A-N. Primary Sertoli cells, transfected with GRp58 specific siRNA (C-H) or control siRNA (I-N), were treated with *at*RA (1 µM) for 2 hrs, either lysed for semi-quantitative RT-PCR (A) or immunoblotting with anti-GRp58 or anti-actin antibody (B), or fixed for immunofluorescence analysis with anti-RARA (C, H, I, and L) and anti-GRp58 (D, G, J, M). Merged images in E, H, K, and N. The intensities of mRNA (A) and proteins (B) of GRp58 and S2 were scanned and graphed with means ± SEM from n=3 independent experiments. RD, relative density. O-Z. Primary Sertoli cells were pretreated with MMTS (1 mM) (O-Q), or MMTS (0.5 mM) (U-W), or DTNB (100 uM) (X-Z) for 30 min, and then treated with *at*RA (1 µM) for 2 hrs, fixed and subjected to immunofluorescence analysis with anti-RARA (O, R, U, and X) and anti-GRp58 (P, S, V, and Y) antibodies. Merged images in yellow color in Q, T, W, and Z. DAPI stained nucleus in blue color. Bar in C, I, O, R, U, and X = 100 µm for C-Z. All experiments were repeated at least three times.

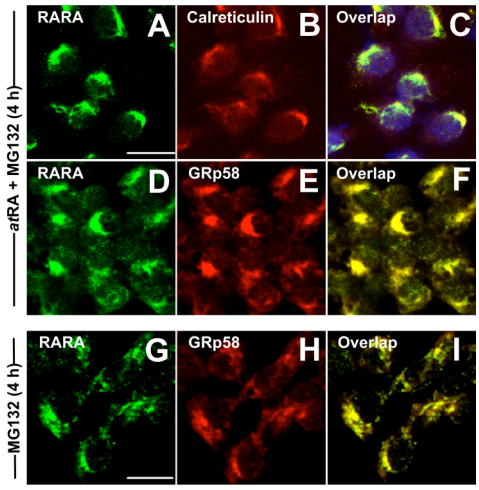

FIGURE 5.5. **MG132 influenced the subcellular localization of RARA and GRp58.** A-I. Primary Sertoli cells were pretreated with MG132 (10 nM) for 30 min and then treated with *at*RA (1 μM) for 4 hrs, fixed and subjected to immunofluorescence analysis with anti-RARA (A, D, and G) and anti-calreticulin (B) or anti-GRp58 (E and H) antibodies. DAPI stained nucleus in blue color. Merged images in yellow color in C, F, and I; . Bar in A and C = 100 μm for A-I.

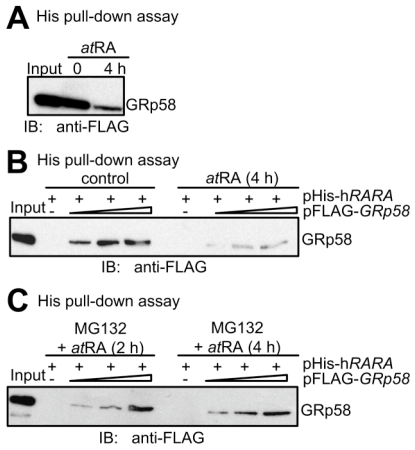

FIGURE 5.6. ***at*RA decreased the interaction between RARA and GRp58 with time and MG132 blocked the *at*RA-induced dissociation of RARA and GRp58.** A-C. COS-7 cells were transfected with pHis-h*RARA* and pFLAG-*GRp58* cDNA expression plasmids (A) or constant amount of pHis-h*RARA* and increasing amounts of pFLAG-*GRp58* cDNA expression plasmids (B-C). After 48 hrs, the cells were pretreated with MG132 (10 nM) for 30 min (C) and then treated with either vehicle or *at*RA (1 μM) for 2 or 4 h, harvested and subjected to His pull-down assay followed by immunoblotting (IB) with anti-FLAG M2 antibody. Input, the same lysate used for pull-down assay. All experiments were repeated at least three times.

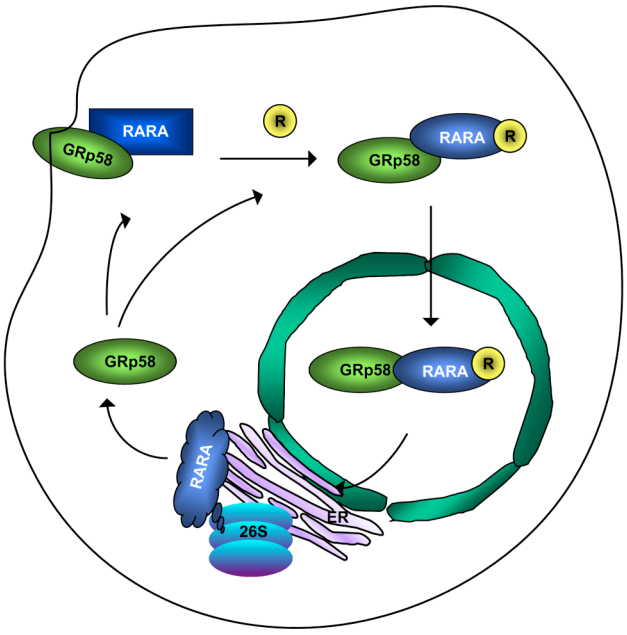

FIGURE 5.7. **A working model for GRp58 regulating the nuclear localization and the subsequent proteolytic processing of RARA.** Binding of *at*RA to RARA (rectangle) induces a conformational change to RARA (oval). R, *at*RA; ER, endoplasmic reticulum; and 26S, proteasomes.

CHAPTER 6

CONCLUSION

CONCLUSION

Spermatogenesis is a complex process occurring in the seminiferous tubule, and it is modulated by many factors both endocrine and paracrine (1, 2). One of the factors, vitamin A, has been shown to be essential for spermatogenesis as well as for the overall survival of animals (3). Animals, fed with a vitamin A-deficient diet, are observed several abnormalities such as testicular degeneration with a majority of tubules having a complete loss of germ cells in testes (3). The action of vitamin A is mediated by retinoid receptors, which are composed of two families of ligand dependent transcription factors, the RAR and RXR (4). Genetic studies have revealed that one of retinoid receptors, RARA, is essential for spermatogenesis as demonstrated by the sterility of *Rara* gene knockout mice (5). The goals of these studies presented herein were to investigate mechanisms by which modulators affect the functions of RARA. More specifically, because the functional role of RARA is as a transcription factor, the focus of this research was to elucidate how RARA-interacting proteins regulate the functions of RARA such as its stability, subcellular localization and transcriptional activity.

Being a transcription factor, RARA mediates the transcription of target genes, but it can be regulated by many factors such as posttranslational modifications and interactions with coregulators. Posttranslational modifications of RARA have been shown to play an important role in regulating its subcellular localization and transcriptional activity (6, 7). Previously, our lab has shown that a short-term exposure of FSH via PKA inhibited *at*RA-induced RARA nuclear localization and, decreased the steady state level of RARA and the RARE-dependent transcription in a mouse Sertoli cell line stably transfected with the FSH receptor cDNA (6). Meanwhile, RARA has been shown to be positively regulated by PKC and MAPK in Sertoli cells independent of the ligand (7). These results suggest that these factors play an important role in modulating the functions of RARA.

YEAST TWO-HYBRID SCREENING

In Chapter 2, to identify RARA-interacting proteins in Sertoli cells, a yeast two-hybrid screening with RARA as the bait and proteins expressed from a Sertoli cell cDNA library as the prey was performed. In this screening, 15 candidates were obtained and two of them, SUMO-2 and GRp58, were further investigated in this thesis.

SUMO-2 MODIFICATION OF RARA

In Chapter 3, we examined SUMO-2 modification of RARA and how sumoylation of RARA led to changes in its protein stability, nuclear localization and transcriptional activity. We found that RARA was modified by SUMO-2 on K399 of RARA in the absence of *at*RA, and on K166, K171 and K399 of RARA in the presence of *at*RA, which agrees with previous studies showing that sumoylation of full-length estrogen receptor alpha (ERA) was ligand-mediated (8), and that sumoylation of androgen receptor on K511 was agonist dependent but not on K318 (9). Presumably, sumoylation on different sites of RARA is due to the fact that ligand binding to nuclear receptor could induce a major conformational change (10, 11).

We demonstrated that sumoylation of RARA affected the nuclear localization and transcriptional activity of RARA. Sumoylation on K399 was required for the cytoplasmic localization of RARA in the absence of *at*RA, however, in the presence of *at*RA, sumoylation on K166 and K171 counteracted the effect of sumoylation of RARA on K399 in regulating the nuclear localization of RARA. The transcriptional activity of RARA was correlated to the nuclear localization of RARA: sumoylation of K399 was required for the wild type transcriptional activity of RARA, whereas, sumoylation of RARA on K166 and K171 inhibited the transcriptional activity of RARA. Additionally, overexpressed SUMO-2 suppressed the transcriptional activity of RARA in a dose-dependent manner. Previously, a SUMO-1 binding deficient c-Jun mutant shows an increased transactivation potential on an AP-1-containing promoter (12); covalently attached SUMO-1 to c-Myb inhibits the transactivation function of c-Myb (13) and overexpression of SUMO leads to the inhibition of $NF_{\kappa}B$-dependent transcription (14). Therefore,

these results are consistent with the inhibition role of sumoylation on target proteins, but, for the first time, we demonstrate that sumoylation on different sites of the same protein has completely different roles in controlling the transcriptional activity of RARA.

We also demonstrated that the *at*RA ligand is an active inducer of RARA sumoylation, whereas, SUMO-2 with *at*RA had a biphasic influence on RARA sumoylation. Both RARA and sumoylated RARA levels were high at first and then declined at low levels of SUMO-2, whereas, their levels stabilized at high levels of SUMO-2. This process required new protein synthesis and proteasome-mediated degradation. Consistently, previous studies showed that sumoylation and proteasomal activity control the transactivation properties of the mineralocorticoid receptor (15), and that ubiquitin-proteasome system is required for the recycling of SUMO-2/3 (16).

In conclusion, we have identified SUMO-2 modification of RARA is a novel posttranslational regulatory mechanism modulating its nuclear localization, transcriptional activity and protein stability. Thus, manipulation of SUMO-2 could be an alternative strategy to control the functions of RARA.

CHARACTERIZATION OF SUMO-2/3 IN TESTES

In Chapter 4, we examined the regulation of SUMO-2/3, whose protein sequences share ~95% similarity, in testes. SUMO-2 and SUMO-3 mRNA were ubiquitously expressed in rat tissues, but, interestingly, both were highly expressed in testes. The free SUMO-2/3 was predominantly expressed in testes throughout development to the adult stage. Consistently, the free SUMO-2/3 was mainly located in the nucleus of germ and Sertoli cells in the early testis development, and after 15 days it was observed in the cytoplasm of germ and Sertoli cells, agrees with previous studies showing that SUMO-1, SUMO-2/3, and UBC9 are present in male germ cells during meiosis and in spermiogenesis (17). Recently, in human patients, abnormal level of SUMO-2/3 has been shown to be tied to azoospermia and hypospermatogenesis or reduction and impairment in the fidelity of spermatogenesis (18), indicating that SUMO-2/3 appears to play a key role in spermatogenesis. Hence, to understand how SUMO-2/3 is regulated in testes seems to be critical for normal spermatogenesis.

We demonstrated that the subcellular localization of SUMO-2/3 was regulated by RA through RARA signaling pathway. The subcellular localization of SUMO-2/3 in VAD rat testes was opposite to that in normal rat testes, confirmed by *in vivo* retinol-injected rats and *in vitro* cultured-testes treated with *at*RA, indicating that the subcellular localization of SUMO-2/3 was regulated by RA. Moreover, the disruption of *Rara* gene in mice cause the subcellular localization change of SUMO-2/3 from the cytoplasm to the nucleus of testicular cells, which was similar to the pattern observed in VAD rats. Moreover, not only *at*RA but also Am580 (RARA specific agonist) influenced the subcellular localization of SUMO-2/3 in primary Sertoli cells, suggesting that RARA signaling pathway regulated the subcellular localization of SUMO-2/3 in Sertoli cells.

Furthermore, we demonstrated that the testicular protein expression of SUMO-2/3 in testes was regulated by other RA signaling pathways, but not by RARA. *at*RA decreased the expression of monomer SUMO-2/3 and increased the SUMO-2/3 complexes in primary Sertoli cells, but neither RARA specific agonist Am580 nor RARA specific antagonist affected the expression of SUMO-2/3. Moreover, the disruption of *Rara* gene in mice didn't influence the protein expression of SUMO-2/3. These data suggest that other RA signaling pathway(s), but not RARA, regulated the protein expression of SUMO-2/3 in Sertoli cells and testes. Previous reports have shown that many types of cellular stresses, including temperature shock, toxic metals, and osmotic and oxidative stress, cause rapid changes in SUMO conjugation (19-21). Similarly, SUMO-1 has been reported that its protein expression is inhibited by LH receptor stimulation during ovulation in mouse granulose cells (22).

In conclusion, the subcellular localization and protein expression of SUMO-2/3 were regulated by different RA signaling pathways in testes. Since hypo SUMO-2/3 and hyper SUMO-2/3 cause problems in spermatogenesis in human patients, it would be promising to further understand the mechanisms how SUMO-2/3 is modulated by RA signaling pathways in testes.

INTERACTION BETWEEN GRP58 AND RARA

In Chapter 5, we demonstrated a significant finding, but not reported previously, that the nuclear localization and the degradation of RARA in the ER are regulated by GRp58, also known as a disulfide isomerase (PDIA3). GRp58 was strongly associated with RARA in the yeast two-hybrid screening as well as in transfected COS-7 cells. Its mRNA and protein were highly expressed in germ and Sertoli cells in testes. Consistently, immunohistochemical analysis showed that GRp58 was present in the testis throughout development to the adult stage. Interestingly, in the early testis development, GRp58 was found mainly located on the membrane and/or in the cytoplasm of germ and Sertoli cells. Around 30 days, it was mainly found in the acrosome structure of the round spermatids, and continued its expression in developing spermatids, which agrees with previous studies (23, 24). In addition, we report a novel finding that GRp58 is present in the nucleus of pre-meiotic germ cells close to the basement membrane.

In Sertoli cells, we observed that the *at*RA ligand was able to mobilize both GRp58 and RARA into the nucleus, which is similar to a previous finding that GRp58, as a membrane receptor of $1,25(OH)_2D_3$, is mobilized from the cytoplasm to the nucleus upon $1,25(OH)_2D_3$ treatment in IEC-6 cells (48). Moreover, when GRp58 was knocked down by RNAi technique, RARA was observed in the cytoplasm of Sertoli cells, even in the presence of *at*RA, suggesting that GRp58 is required for the nuclear localization of RARA. This resembles previous studies showing that glucocorticoid receptor translocation to the nucleus and mobility within the nucleus require the presence of the Hsp90 machine (25, 26). We also observed the nuclear localization of RARA and GRp58 was disrupted by the pretreatment with sulfhydryl modifying agents MMTS and DTNB. Previous studies have shown that sulfhydryl groups in the ligand-binding domain of RARA are involved in ligand-receptor interactions, and the ligand-binding activity of RARA is markedly decreased by sulfhydryl modifying agents such as MMTS and DTNB, which modify the cysteine residues (27, 28). Thus, taken together, it appears that cysteine residues are important for the *at*RA-dependent nuclear localization of RARA, and the thiol oxidoreductase activity of GRp58 may be required for the thiol exchange reactions with the cysteine residues of RARA for enhancing the *at*RA binding to RARA.

Furthermore, we also demonstrated that before GRp58 could de-couple from RARA, it had to accompany RARA to the ER for degradation. In the absence of *at*RA, GRp58 with RARA were co-localized in the cytoplasm of Sertoli cells. Upon *at*RA treatment, GRp58 with RARA were found co-localized to the nucleus of Sertoli cells in 2 hrs, but the majority of RARA were located in the ER, while GRp58 was localized to the cytoplasm in 4 and 8 hrs. Interestingly, in the presence of proteasome inhibitor and *at*RA, GRp58, RARA and calreticulin, an ER marker, were observed co-localized in the ER, suggesting that the inhibition of proteasome-mediated degradation pathway caused the retention of GRp58 and RARA in the ER. Consistently, the level of GRp58 associating with HIS-RARA in the cells treated with *at*RA at 4 hrs was significantly less compared to that in the untreated cells. Moreover, the amount of GRp58 copurified with HIS-RARA was significantly higher in the presence of both *at*RA and MG132 at 4 hrs than that in *at*RA alone treated samples. These results suggest that *at*RA induced the degradation of RARA, as expected, but also led to dissociation of GRp58 from RARA. Based on these findings, our speculation is that upon *at*RA treatment, GRp58 accompanies RARA to the ERAD machinery, the cells's protein quality control system. Whether calreticulin or calnexin serve as a conduit for RARA degradation is not known, although it was reported previously that calreticulin physically interacts with RARA, as shown by co-immunoprecipitation (29). More intriguingly, a corepressor, N-CoR, associated with PML-RARA, a fusion protein of promyelocutic leukemia (PML) and RARA that causes acute promyelocytic leukemia (APL), was shown undergo the ERAD, including ubiquitylation of N-CoR via an ubiquitin-conjugating enzyme, Ubc6 (30). This report suggests that a reduction in N-CoR protein levels in the nucleus could be partially responsible for uncontrolled growth in APL.

In conclusion, we have identified GRp58 is a novel molecular chaperone for RARA and it participates in the nuclear localization and the degradation of RARA in the ER. Thus, in the study we demonstrate that the manipulation of GRp58 could be an alternative mechanism to modulate the functions of RARA in Sertoli cells.

SUMMARY, FUTURE WORK AND TRANSLATIONAL RESEARCH

In conclusion, these results establish the regulations of RARA by SUMO-2 modification and interaction with molecular chaperone GRp58. We demonstrate that SUMO-2 modification of RARA is a novel posttranslational modification of RARA. The *at*RA ligand and the amount of SUMO-2 are two key components regulating sumoylation of RARA. SUMO-2 modification of RARA affects its stability, nuclear localization and transcriptional activity. In addition, we show that the subcellular localization and protein expression of SUMO-2/3 were regulated by different RA signaling pathways in testes. These results suggest that the relationship among RA, RARA and SUMO-2 is finely tuned. Finally, our results also demonstrate that GRp58 is a novel molecular chaperone for RARA and participates in the nuclear localization and the degradation of RARA in/on the ER.

Based on all these studies, we proposed an overall working model (Fig. 6.1) showing how RARA is regulated by SUMO-2 and GRp58. In the absence of *at*RA, RARA, which is modified by SUMO-2 on K399, interacts with GRp58 in the cytoplasm. SENP6 may associate with or near the sumoylated RARA and be responsible for the de-sumoylation of RARA from K399 to keep the level of the sumoylated RARA low in Sertoli cells. Upon *at*RA binding to RARA, a conformational change occurs and reveals multiple sumoylation sites on RARA to be modified by SUMO-2. Meanwhile, GRp58, which associates with RARA, accompanies RARA to translocate into the nucleus, while SUMO-2 may be de-conjugated by SENP6 which was co-localized with RARA in the nucleus upon *at*RA treatment. After transcription, GRp58 may delivery RARA either through ER or through nuclear pore to utilize ER associated degradation machinery (ERAD) for RARA degradation. When RARA is degraded, GRp58 may be able to dissociate from RARA and then translocate back to the cytoplasm for the next cycle.

To further confirm this model, firstly we need to determine whether RARA, GRp58 and SUMO-2 could form a complex in the cytoplasm and the molecular weight of the complex would be increased upon *at*RA treatment, because more sumoylation sites are revealed by *at*RA in Sertoli cells, using immunoprecipitation. Secondly, we also need to investigate whether the disruption of SENP6 would increase the level of the sumoylated RARA and decrease the transcriptional activity of RARA by RNAi

technique, by knocking down endogenous SENP6 or mutating the catalytically active site of SENP6 in SENP6-overexpressed cell line. Additionally, since SUMO-1 has been shown to be compensated by SUMO-2 and SUMO-3 in testes, as demonstrated by *Sumo-1*$^{-/-}$ knockout mice (31), in future, the disruption of endogenous SUMO-2 using RNAi technique in germ cells and Sertoli cells would be a better way to help us understand the physiological role of SUMO-2 in testes.

We also need to determine whether the thiol oxidoreductase activity of GRp58 help to enhance the ligand binding to RARA, important for the nuclear localization of RARA, by mutating the cysteine residues in the LBD of RARA , which are important for ligand binding (27), and/or by mutating the two thioredoxin active sites of GRp58. Furthermore, we also need to elucidate the mechanism how RARA is degraded by ER associated degradation machinery (ERAD). In future, two candidates Ube2j1 and Ube2j2, two E2 ubiquitin conjugation enzymes, known to be involved in the ERAD (32), will be investigated by immunofluorescence to determine whether RARA is co-localized with these two enzymes and by RNAi technique to determine whether the disruption of these enzymes would affect the stability of RARA.

Our lab has shown that RARA in Sertoli cells is critical for the germ cell development through meiosis. Studies present herein suggest that the transcriptional activity of RARA is inhibited by hyper SUMO-2, and the dynamic SUMO-2 conjugation and de-conjugation are critical for the activity of RARA. In human patient, hyper SUMO-2/3 causes azoospermia and hypospermatogenesis (33). Therefore, in clinic, targeting SUMO-2 and SUMO-3 or their activating and/or conjugating enzymes by inhibitors or RNAi-based drug in Sertoli cells could be a promising strategy to cure male infertility. For the purpose of contraception, since hypo SUMO-2/3 causes the problems in spermatogenesis (33), we could manipulate the level of SUMO-2 and SUMO-3 in Sertoli cell by targeting SUMO-2/3 de-conjugating enzymes such as SENP6 via designed inhibitors or RNAi-based drugs. Additionally, we also could disrupt the function of GRp58 by GRp58 antibody or inhibitor to inhibit the activity of RARA in Sertoli cells, resulting in the disruption of germ cell meiosis.

Overall, we are the first to report that RARA is modified by SUMO-2 and GRp58, which affect its nuclear localization, transcriptional activity and protein stability. Our results suggest that the manipulation of either SUMO-2 or GRp58 could be alternative strategies to modulate the functions of RARA in Sertoli cells.

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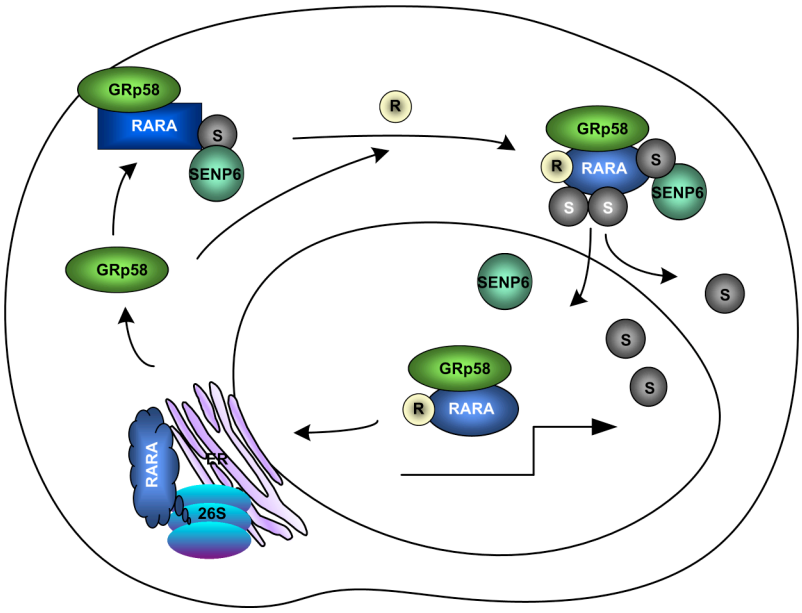

FIGURE 6.1 **A working model for the regulation of RARA by SUMO-2 and GRp58.** Binding of atRA to RARA (rectangle) induces a conformational change to RARA (oval). S, SUMO-2; R, *at*RA; ER, endoplasmic reticulum; and 26S, proteasomes.