Association of NASP with HSP90 in Mouse Spermatogenic Cells: Stimulation of ATPase Activity and Transport of Linker Histones into Nuclei

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Running title: tNASP-H1t in germ cells

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Summary

NASP (nuclear autoantigenic sperm protein) is a linker histone binding protein found in all dividing cells that is regulated by the cell cycle (9) and in the nucleus linker histones not bound to DNA are bound to NASP (8). In mouse spermatogenic cells tNASP binds the testis specific linker histone H1t. Utilizing a cross-linker, DTSSP, and mass spectrometry, we have identified HSP90 (heat shock protein 90) as a tNASP binding partner. In vitro assays demonstrate that tNASP’s association with HSP90 stimulated the ATPase activity of HSP90 and increased the binding of H1t to tNASP. HSP90 and tNASP are present in both nuclear and cytoplasmic fractions of mouse spermatogenic cells, however, HSP90 bound to NASP only in the cytoplasm.

In vitro nuclear import assays on permeabilized HeLa cells demonstrate that tNASP, in the absence of any other cytoplasmic factors, transports linker histones into the nucleus in an energy and NLS (nuclear localization signal) dependent manor. Consequently we hypothesize that in the cytoplasm linker histones are bound to a complex containing NASP and HSP90, whose ATPase activity is stimulated by binding NASP. NASP-H1 is subsequently released from the complex and translocates to the nucleus where the H1 is released for binding to the DNA.
Introduction

Linker histones (H1s) bind to DNA in the nucleosomes of chromatin and influence the formation of chromatin fibers, gene transcription, nucleosome spacing, chromatin remodeling, and cell cycle progression (1, 2, 3, 4, 5). Studies on linker histones in the cell nucleus indicate that there is a constant exchange of H1s with their DNA binding sites (6, 7) and that H1s not bound to DNA are bound to the histone-binding protein NASP (8). NASP is a histone-binding protein found in all dividing cells in either a somatic/embryo (sNASP) or testis/embryo (tNASP) form (9, 10, 11). Overexpression of tNASP affects progression through the cell cycle and Alekseev et al. (8) recently postulated that a dynamic equilibrium exists between H1-NASP complexes and H1 bound to DNA. Previous studies on NASP (9) demonstrated that in vivo the somatic linker histones H1a-H1e (see 12 for mouse histone H1 nomenclature) were co-precipitated with NASP from somatic cell lysates, indicating that NASP is not selective for H1 subtypes.

Of the several different subtypes of H1 histones, H1a,b,c,d,e are found in most cells while H1t is restricted to the testis. H1t is expressed during spermatogenesis, representing as much as 50% of the total H1 histones in pachytene spermatocytes (13, 14, 15), and is preceded by tNASP expression in preleptotene spermatocytes. Although their exact function during spermatogenesis is unknown, gene-targeting experiments (16) have shown that the absence of H1t and H1a in double null mice does not compromise spermatogenesis because compensation by H1c, H1d, and H1e most likely maintained normal spermatogenesis in spite of a reduced H1/nucleosome ratio.
(16). Under these conditions the major H1 histone binding activity of tNASP could have shifted from H1t to H1c, H1d and H1e in double null mice. Unfortunately neither the relationship between tNASP, H1t and other proteins nor the ability of tNASP to transport H1s into the mammalian cell nucleus has been characterized. Therefore we have studied tNASP in spermatogenic cells to understand more about the role of NASP-H1t complexes and now report that H1t binds tNASP in mouse germ cells in a complex in the cytoplasm that contains HSP90, which facilitates the initial loading of linker histones to tNASP. Moreover, tNASP can transport both H1t and somatic linker histones into nuclei.

**Experimental Procedures**

All chemicals and reagents used in this study were molecular biology grade. Restriction enzymes were purchased from Roche Diagnostics (Roche Molecular Biochemicals, Indianapolis, IN). Purification of plasmid DNA and PCR products were carried out using QIAprep Miniprep and QIAquick PCR purification kits (Qiagen, Valencia, CA) and sequencing was performed at the UNC-CH automated sequencing facility. Rabbit anti-histone H1 (FL-219) polyclonal antiserum and mouse monoclonal IgG2a anti-HSP90 (F-8) antiserum were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-H1t antibody was a gift from Dr. M. Meistrich (Department of Experimental Radiation Oncology, M. D. Anderson Cancer Center, University of Texas, Houston, TX). Rabbit antisera to the N-terminal of NASP (nucleotides 96-1099) were prepared as previously described (9). Rabbit antisera to full length recombinant NASP (nucleotides 92-2405) were made by Bethyl Laboratories (Montgomery, TX). Somatic
linker histones from calf thymus were purchased from Roche Molecular Biochemicals (Indianapolis).

The entire coding sequence of mouse tNASP (nucleotides 92-2406, GenBank accession number AF034610) was amplified from mouse testis Quick-clone cDNA (Clontech, Palo Alto, CA) as described previously (8). A tNASP deletion mutant (NASP-ΔTPR; nucleotides 92-1590), that lacked all tetratricopeptide repeats (nucleotides 1591-1891) was PCR-amplified, cloned into pCR® T7/CT-TOPO vector (Invitrogen, Carlsbad, CA), expressed and purified identically to the full-length construct (8). Preparation of the NLS deletion mutant (NASP-ΔNLS) has been described previously (8).

Isolation, separation, and short-term culture of spermatogenic cells

Isolation, separation, and short-term culture of spermatogenic cells were carried out as previously described (17, 18). Cells were grown in plastic flasks for 16-18 hours to allow Sertoli cells and other somatic cells to adhere to the bottom of the flask and spermatogenic cells were collected from the supernatant.

Harvested mouse spermatogenic cells were washed in PBS and Earle’s Balanced Salt Solution (5.3 mM KCl, 117 mM NaCl, 26 mM NaHCO₃, 1 mM NaH₂PO₄ and 5.6 mM glucose) and resuspended in 10 volumes of RBS buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂) containing 1 mM phenylmethylsulfonylfluoride (PMSF), and protease inhibitor cocktail (Cat #8340, Sigma St. Louis, MO). Nuclei were prepared essentially as described (19). After incubation on ice for 10 minutes cells were transferred to a pre-chilled glass Dounce-type...
homogenizer and subjected to 10-12 quick strokes of the pestle. Nuclei were removed from the
cytoplasmic fraction by centrifugation at 1000 × g for 3 minutes, washed with PBS and sonicated
for 20 seconds. The supernatant (nuclear fraction) was cleared by centrifugation for 10 minutes
at 1000 × g. Microscopic examination of the nuclei and Western blotting (staining for histones)
of nuclear and cytoplasmic fractions confirmed their separation.

In vitro nuclear transport assay

The nuclear import assay was performed on permeabilized HeLa cells as described
previously (20). Cells were grown on 22 × 22 mm glass cover slips in six well plates (Corning,
Corning, NY) and washed in ice-cold import buffer (20 mM Hepes, pH 7.3, 110 mM potassium
acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT, 1 µg/ml each of aprotinin,
leupeptin, and pepstatin). Cells were permeabilized in ice-cold import buffer containing 40
µg/ml digitonin (40 mg/ml stock solution in dimethyl sulfoxide; Calbiochem, San Diego, CA) for 8
min. Digitonin containing buffer was replaced by ice-cold import buffer and the cells washed in
several changes of import buffer for 30 min with gentle rocking. The complete import mixture
contained 50% (v/v) cytosol (the cytoplasmic fraction of HeLa cells centrifuged at 60 000 × g,
total protein concentration 10 mg/ml), 1 mM adenosine-5’-triphosphate, 5 mM creatine
phosphate, 20 units/ml creatine phosphokinase (Calbiochem, San Diego, CA), and 10 µM biotin
labeled histone H1t. Mouse tNASP, or NASP-ΔNLS was added to a final concentration of 5
µM. The cover slips were inverted over 150 µl of complete import mixture on a sheet of parafilm in
a humidified plastic box and incubated at 37°C for 30 min. The transport reaction was stopped by
fixation in 3% formaldehyde for 15 min. Cover slips were incubated in Texas Red Avidin D
(Vector Laboratories, Burlingame, CA) for 1 hour, briefly washed in PBS and incubated in DAPI
Nucleic Acid Stain (Molecular Probes, Eugene, OR) for 2 min. Cover slips were mounted in
ProLong Antifade (Molecular Probes, Eugene, OR) and examined with a Zeiss Axiophot as
previously described (21).

**Chromatography**

Immunoaffinity chromatography was carried out as described previously (8). For the
preparation of NASP enriched fractions from testis, lysates of 50 mouse testes (Pel-Freez®
Biologica, Rogers, AR) were prepared in 2 ml of MPER reagent (Pierce, Rockport, IL) with
Protease (Cat #8340, Sigma, St. Louis, MO) and Proteasome (Cat #539160, Calbiochem, San
Diego, CA) inhibitors added. Cells were vortexed thoroughly and rocked 10 min at room
temperature. Debris was removed by centrifugation (10 min, 12,000 rpm) and the supernatant
applied to a 21 × 600 mm Bio-Sep SEC-S 3000 size exclusion HPLC column equipped with a
75 × 21.2 mm pre-column (Phenomenex, Torrance, CA) in 10 mM phosphate buffer, pH 7.0,
252 mM NaCl. 1650 µl were injected per run at a flow rate of 7 ml/min and fractions collected
every 0.25 minutes for 27 minutes. 500 µl aliquots of the collected fractions were concentrated in
Amicon (Beverly, MA) microcentrifuge filters (molecular weight cut off =10,000 Daltons),
boiled with SDS-sample buffer containing β-mercaptoethanol and analyzed by Western
blotting. NASP positive fractions were pooled (fig. 2A).

Linker histones were purified from mouse testes (Pel-Freez® Biologicals, Rogers, AR)
according to the method of Brown and Sittman (22). Lyophilized histones were dissolved in 0.1% trifluoroacetic acid and applied to a C-18 reverse phase column (Waters, Milford, MA; Delta-Pak, 15 µm, 300 Å, 3.9 × 300 mm) as described previously (8, 9). H1t fractions were pooled and used for further experiments.

Mass spectrometry identification of tNASP binding partners

Water-soluble cross-linker DTSSP (3,3’-dithiobis-(sulfosuccinimidylpropionate), 2mM; Pierce, Rockford, IL) with a spacer arm length of 12 Å was added to the Bio-Sep SEC-S 3000 column NASP pooled fraction (described above), incubated (room temperature, 30 min) and analyzed by non-reducing SDS-PAGE. Western blots probed with anti-NASP antibody identified a high molecular weight NASP positive band. A strip of gel containing the high molecular weight NASP positive band was excised from a second identical gel, incubated in 50 mM DTT (dithiothreitol (Sigma, St.Louis, MO, 37°C, 30 minutes), placed horizontally into a well of a 10% acrylamide gel (1 mm thick) and separated in the second dimension in the presence of β-mercaptoethanol. The resulting gel was stained by 0.01% BioRad R-250 Coomassie (Bio-Rad Laboratories, Hercules, CA) in 10% acetic acid overnight. Protein staining bands located below the position of the noted high molecular weight NASP positive band (~200 kDa) were excised and prepared for MALDI/TOF analysis. Digestion of the selected bands was accomplished using the Genomic Solutions ProGest with Sequencing Grade Modified Trypsin (Promega, Madison, WI). The resultant peptide solution was then lyophilized and reconstituted in 45% methanol: 45% water: 10% formic acid. This solution was combined on the MALDI
target with a saturated matrix solution of alpha-cyano-4-hydroxy-cinnamic acid. MALDI TOF and MALDI TOF/TOF were performed on the AB 4700 Voyager - Proteomics Discovery System (Applied Biosystems, Foster City, CA). The resulting peptide peaks were searched against the MSDB and NCBI databases using the MASCOT search engine of GPS Explorer software. Mass spectrometry identification was done in the UNC-CH Proteomics Core Laboratory.

**ATPase assay**

The amount of ATP in solution was detected by the ENLITEN® ATP Assay System Bioluminescence Detection Kit (Promega, Madison, WI). Bioluminescence was measured on a TD-20/20 Luminometer (Turner BioSystems, Sunnyvale, CA). Assays were performed in 40 mM HEPES, pH 7.5, 5 mM MgCl₂, and 10⁻⁸ M ATP (Calbiochem, San Diego, CA). Typical protein concentrations were 0.2 µM for HSP90 and albumin; 0.2 µM for tNASP unless otherwise indicated. The temperature was set to 37°C. Background (nonspecific) readings were subtracted from the total activity. Relative light units (RLU) were normalized for comparing the results from different experiments. As ATPase activity increases the RLU decrease. Significance was determined by the Student’s t-Test.

**Co-immunoprecipitation**

For binding experiments reverse phase HPLC purified H1t was biotin-labeled and mixed with recombinant tNASP (10 µg) in 500µl volume of 40 mM HEPES, pH 7.7, 5 mM MgCl₂ and incubated for 1 h at 37°C with gentle shaking. As required by the experiment, HSP90 (2 µg)
and/or ATP (1 mM) were added. Precipitations were carried out with affinity-purified rabbit anti-recombinant NASP antibodies. Antibody-protein complexes were collected by Ultra Link® Immobilized Protein A/G (Pierce, Rockford, IL) and eluted by boiling in SDS-PAGE sample buffer containing β-mercaptoethanol. Samples were separated in 10-20% SDS-polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) by electrophoresis and the amount of co-immunoprecipitated H1t calculated from background-subtracted images using Gel Expert Software (Nucleotech, San Carlos, CA).

**Results**

**Association of H1t histones with tNASP**

Isolation of NASP from mouse testis lysates by affinity chromatography with anti-NASP antibodies resulted in co-purification of H1 histones. Previous studies on NASP (9) demonstrated that the somatic linker histones were co-precipitated with NASP. The presence of linker histone H1t was identified by probing Western blots with specific H1t antibody (Fig. 1, lane 3). Western blot analysis of the H1t co-purified with tNASP and H1t histones extracted from the mouse testis and isolated by reverse phase HPLC indicated that they had identical SDS-PAGE profiles (Fig. 1, lane 4).

**Identification of tNASP binding partners**

To identify additional binding partners that may be complexed with tNASP in mouse germ cells we used size exclusion chromatography and gel electrophoresis to isolate tNASP complexes. tNASP complexes were identified by SDS-PAGE and Western blot analysis with
anti-NASP antibodies following size exclusion HPLC separation of a mouse testis lysate on a Bio-Sep SEC-S 3000 column (Fig. 2A). NASP complexes were pooled and cross-linked with DTSSP. Non-reducing SDS-PAGE analysis of the cross-linked complexes indicated the appearance of an approximately 200 kDa NASP positive band (asterisk Fig. 2B). This cross-linked complex was cleaved by incubation of the gel in DTT and the gel lane subsequently placed horizontally and electrophoresed in the second dimension. Immediately under the reduced NASP complex two major protein-staining bands were revealed (bands 1 and 2, Fig. 2C).

Initial identification of band 1 by MALDI TOF (Fig. 3A) and additional confirming MALDI TOF/TOF analysis of its peaks 1485.82 and 1187.67 (Fig. 3B, C) identified this band as an endoplasmic reticulum protein 99 precursor (accession number A29317; tumor rejection antigen gp96). This protein belongs to the heat shock protein 90 family and has a histidine kinase-like ATPase domain. Band 2 (Fig. 2C) was identified as mouse heat shock protein 90-alpha (accession number P070901; HSP86-1, tumor specific transplantation 86 kDa antigen, TSTA) with MALDI TOF/TOF analysis of peaks 1264.59 and 1513.77 (Fig. 4A, B, C). Heat shock protein 90 is a molecular chaperone with ATPase activity.

Affinity chromatography with anti-NASP antibodies confirmed the interaction between tNASP and HSP90 in mouse testis lysates (Fig. 5A). Moreover, as expected from the cross-linking studies of the HPLC (Bio-Sep SEC-S 3000 column) tNASP complexes, HSP90 was present in significant amounts (Fig. 5A). The distribution of tNASP and HSP90 in nuclear and cytoplasmic compartments was also studied to confirm the interaction between tNASP and
HSP90 in mouse germ cells. As shown in figure 5B, tNASP and HSP90 appear unequally distributed between nuclear and cytoplasmic fractions of mouse spermatogenic cells. Somatic NASP was present in small amounts in both nuclear and cytoplasmic fractions (Fig. 5B) and probably represents a contaminant from non-spermatogenic cells in testis. The quality of the nuclear and cytoplasmic preparations was monitored by the presence of histone H1 staining (Fig. 5C). Affinity chromatography with anti-NASP antibodies confirmed that the nuclear and cytoplasmic fractions of mouse germ cells contain tNASP (Fig. 5D, lanes 1,2). However, HSP90 co-purified with tNASP only from the cytoplasmic fraction (Fig. 5D, lane 4) and HSP90 alone did not bind to the anti-NASP affinity column (Fig. 5D, lane 5).

**ATPase activity of HSP90 is stimulated by tNASP**

Non-activated mammalian HSP90 has very low ATPase activity (23). The HSP90 ATPase activity, in the presence of ATP, was not significantly different from ATP alone or ATP + tNASP alone or ATP + HSP90 + bovine serum albumin (Fig. 6A). However, in the presence of tNASP the ATPase activity of HSP90 increased significantly (Fig. 6A; p< 0.03) and increasing amounts of tNASP (0.1, 0.2 and 0.4 µM) resulted in increasing HSP90 ATPase activity (Fig. 6B).

The presence of a tetratricopeptide repeat (TPR) domain has been reported from a variety of proteins to interact with HSP90 (24). NASP contains TPR-domains; therefore we tested a mutant tNASP lacking the TPR sites (NASP-ΔTPR). When the HSP90-ATP reaction mixture was incubated with NASP-ΔTPR the HSP90 ATPase activity was strongly activated, although
somewhat lower in activity than with intact tNASP (Fig. 6C). The tNASP TPR domains are apparently not required for the activation of HSP90 ATPase activity.

**H1t binding in the presence of HSP90**

Previous studies demonstrated that NASP binds somatic linker histones (9) and in the present study we have demonstrated that tNASP binds mouse testis specific histone H1t. Because NASP stimulates HSP90 ATPase activity, we tested whether or not NASP binding H1t would increase in the presence of HSP90 and ATP. Figure 7 demonstrates that the binding of H1t to NASP significantly increases in the presence of HSP90 and ATP (p < 0.04).

**Nuclear transport of linker histones by tNASP**

Protein nuclear import requires soluble cytoplasmic factors (20) and linker histones may use importin β and importin 7 heterodimers for nuclear transport (25). However, tNASP has both a functional nuclear localization signal (10) and functional linker histone binding sites (9). Therefore we tested the ability of tNASP to transport biotin-labeled H1t and somatic H1 histones into the nucleus utilizing an in vitro nuclear transport assay (20). As shown in figure 8A, B, G, Table 1, and as previously described (25), permeabilized HeLa cell nuclei do not transport H1 histones into the nucleus without the presence of cytoplasmic factors. Strikingly, tNASP (Table 1) can completely replace the cytoplasmic factors and transport both H1t (Fig. 8C) and somatic H1 histones (Fig. 8H) into the nucleus. Transport by tNASP does not proceed at 4°C (Fig. 8D), in the absence of ATP (Fig. 8E), or in the absence of the nuclear localization signal (Fig. 8F), indicating that transport is an energy dependent process that requires the NLS.
**Discussion**

In this study we have demonstrated that the histone-binding protein tNASP binds the testis specific linker histone H1t and that tNASP’s association with HSP90 (identical to HSP86-1, accession number P07901) stimulated the ATPase activity of HSP90 and the binding of H1t to tNASP. We have shown that tNASP and HSP90 are present in both nuclear and cytoplasmic fractions of mouse germ cells; significantly HSP90 is bound to NASP only in the cytoplasm, leading to the conclusion that one function of HSP90 may be to chaperone the proper folding of NASP in order to bind linker histones. The timing of the stage specific expression of HSP90, tNASP and H1t supports the conclusion that the tNASP-H1t-HSP90 complex forms in the cytoplasm because HSP90 is expressed beginning on day ten postpartum and approximately two days before the rapid increase in tNASP expression (26; Mammalian Reproductive Genetics Database: [http://mrg.genetics.washington.edu/](http://mrg.genetics.washington.edu/)). Expression of HSP90 and tNASP precede the expression of H1t in pachytene spermatocytes (13, 14, 15).

The activation of HSP90 ATPase activity by tNASP was similar to that reported for the specific activator Aha1 in yeast (27) and C143 in human cells (28). Surprisingly tNASP activation of HSP90 did not depend upon binding to any of the three TPR sites found in the C-terminus of NASP. TPR sites have previously been reported (29) as protein-protein interaction sites between heat shock proteins and a number of functionally unrelated proteins. Our observation that NASP-HSP90 complexes occur only in the cytoplasm implies that there is a mechanism to release NASP from the complex. HSP90 can auto-phosphorylate serine (30) or threonine (23) and this has been proposed to control chaperone binding (31, 32, 33, 34), therefore
phosphorylation of HSP90 may regulate tNASP binding (35).

In addition to HSP90 ATPase activation and increased H1t binding, this study demonstrated that tNASP transports H1t as well as somatic H1 histones into the nucleus. Linker histones have not been reported to be free in the cytoplasm and with the help of HSP90 ATPase activity, are likely to be immediately bound by tNASP upon completion of their synthesis in the cytoplasm. Released from the HSP90 complex, tNASP-H1t would then translocate to the nucleus using the NASP NLS, which has been shown to be necessary and sufficient for NASP transport into the nuclei of *Xenopus* oocytes (10). Linker histones do not have an NLS, however H1 histones can translocate into the nucleus in the presence of a cytoplasmic factor(s) (36) or importin β and importin 7 heterodimers (25); processes that are both temperature and ATP dependent. Similarly tNASP, in the absence of any other cytoplasmic factors, transports linker histones into the nucleus in an energy and NLS dependent manner. Consequently we hypothesize that after the synthesis of linker histones in the cytoplasm they are bound to a complex containing NASP and HSP90, whose ATPase activity is stimulated by binding NASP. NASP-H1 is subsequently released from the complex and translocates to the nucleus where the H1 is released for binding to the DNA (8).

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Footnotes

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The abbreviations used are: NASP, nuclear autoantigenic sperm protein; tNASP, testis/embryo form of NASP; sNASP, somatic/embryo form of NASP; HSP90, heat shock protein 90; NLS, nuclear localization signal; H1, linker histone; H1t – testis form of H1; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonylfluoride; EGTA, ethylene glycol bis (2-aminoethyl ether)-N,N,N’N’-tetraacetic acid; DTT, dithiothreitol; ATP, adenosine triphosphate; DTSSP, 3,3’-dithiobissulfosuccinimidyl propionate; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MALDI, matrix assisted laser desorption/ionization; TOF, time of flight; RLU, relative light units; TPR, tetratricopeptide repeat.
**Figure Legends**

**Fig. 1.** Western blot analysis of tNASP and H1t in the eluates from anti-NASP affinity chromatography of mouse testis lysates

Lane 1. Mouse testis lysate loaded onto the affinity column, probed with anti-NASP antibodies

Lane 2. Eluate from the affinity column, probed with anti-NASP antibodies

Lane 3. Eluate from the affinity column, probed with anti-H1t antibodies

Lane 4. Reverse phase HPLC purified mouse H1t probed with anti-H1t antibodies

**Fig. 2.**

A. Size exclusion HPLC separation of tNASP from a mouse testis lysate; fractions (14.5-16 min; patterned area) were identified by Western blotting as NASP positive fractions.

B. Pooled tNASP positive fraction separated by non-reducing SDS-PAGE with or without cross-linker DTSSP added to a final concentration of 2mM. Asterisk shows the position of the cross-linked (~200 kDa) tNASP positive band.

C. The lane containing cross-linked tNASP was cleaved by incubation in 50mM DTT, placed horizontally onto a 10% acrylamide gel and separated in the second dimension (reducing). Protein staining bands located below the tNASP positive band (arrows) were excised, digested with trypsin and identified by MALDI-TOF and MALDI-TOF/TOF.

**Fig. 3.**

A. Matrix-assisted laser desorption ionization analysis (MALDI-TOF) of tryptic
peptides recovered from spot # 1 of the second dimension gel of tNASP binding partners demonstrates peaks typical for tumor rejection antigen gp96.

B, C. Two peaks, m/z 1485.82 and 1187.67 (from 3A) sequenced by Tandem Mass Spectrometry (MALDI-TOF/TOF) confirmed the tumor rejection antigen gp96 sequence.

Fig 4. A. Matrix-assisted laser desorption ionization analysis (MALDI-TOF) of tryptic peptides recovered from spot # 2 of the second dimension gel of tNASP binding partners demonstrates peaks typical for HSP90.

B, C. Two peaks, m/z 1264.59 and 1513.77 (from 6 A) sequenced by Tandem Mass Spectrometry (MALDI-TOF/TOF) confirmed the HSP90 sequence.

Fig 5. Association of tNASP with HSP90 in mouse germ cells.

A. Lanes 1-2: Eluate from the anti-NASP antibody affinity column loaded with a mouse testis lysate, probed with rabbit anti-NASP antibody (lane 1) and anti-HSP90 antibody (lane 2);

Lanes 3-4: Size exclusion HPLC tNASP enriched fraction probed with rabbit anti-NASP antibody (lane 3) and anti-HSP90 antibody (lane 4).

B. Mouse germ cell nuclear and cytoplasmic fractions

Lanes 1-2: Probed with rabbit anti-NASP antibody

Lanes 3-4: Probed with rabbit anti-HSP90 antibody
Lanes 5-6: Amido black stain. Lanes were loaded with equal amounts of protein.

C. Mouse germ cell nuclear and cytoplasmic fractions probed with anti-H1 antibody.

Lanes 1-2: No H1 is detected in the cytoplasmic fraction, demonstrating the effectiveness of the separation.

Lane 3 - commercial calf thymus H1 probed with anti-H1 antibody

D. Eluate from the anti-NASP antibody affinity column loaded with either a mouse testis cytoplasmic or nuclear fraction.

Lane 1-2: Probed with rabbit anti-NASP antibody

Lane 3-4: Probed with anti-HSP90 antibody

Lane 5: Control blot of HSP90 loaded onto the affinity column and eluted. Probed with anti-HSP90 antibody.

Lanes 6-7: Amido black stain. Lanes were loaded with equal amounts of protein.

Cyt=cytoplasmic fraction; nucl=nuclear fraction

Fig. 6. ATPase activity of HSP90

A. ATPase activity of HSP90 is stimulated by tNASP.

ATP alone (◊) and ATP +tNASP (n)

ATPase activity of HSP90+ATP (²)

ATPase activity of HSP90+ATP in presence of albumin (ε)

ATPase activity of HSP90+ATP significantly increases (p< 0.03) with tNASP () present
B. Increasing concentrations of tNASP stimulate increasing ATPase activity of HSP90.

ATP-HSP90 mixture was incubated with 0 (†), 0.1 (n), 0.2 (²) and 0.4 µM () of tNASP.

C. Absence of tNASP TPR sites does not significantly reduce ATPase activity of HSP90.

ATP only (†); HSP90 + ATP (n); ATP-HSP90 mixture incubated with 0.4 µM of tNASP (²); ATP-HSP90 mixture incubated with 0.4 µM of tNASP-ΔTPR, mutant lacking the TPR sites ()

**Fig.7.** Co-immunoprecipitation of biotinylated H1t and tNASP by rabbit anti-NASP antibody with or without HSP90 and ATP. Samples were separated by 10-20% SDS-PAGE and the amount of co-immunoprecipitated H1t calculated from background-subtracted images with gel imaging software. The relative amount of H1t is the average from 4 experiments.

Column 1: Non-specific background binding

Column 2: Non-specific binding of H1t + HSP90

Column 3: Binding of H1t to tNASP is significantly different (p<0.0007) from the non-specific binding of H1t to HSP90 (column 2).

Column 4: Binding of H1t to tNASP in the presence of HSP90. The presence of HSP90 does not significantly increase H1t binding (column 3).

Column 5: Binding of H1t to tNASP in the presence of HSP90 and 1mM ATP. The presence of ATP significantly (p<0.04) increases H1t binding to tNASP. Error bars represents ± S.D.
Asterisk (*) shows significantly different amounts.

**Fig. 8.** Nuclear transport of biotinylated linker histones in permeabilized HeLa cells. Top panels show dual fluorescence of H1t (A-F) and H1s (G-H) detected by Texas-Red avidin and nuclei by DAPI stain and bottom panels show only DAPI nuclear stain. All images were recorded with a Zeiss AxioCam through a 40x plan-neofluar 0.75 NA objective. Scale bar is 20 µm for all images.

A. H1t histones at 37°C are not detected within the nuclei.
B. H1t histones at 37°C in the presence of cytoplasmic extract are detected within nuclei.
C. H1t histones at 37°C in the presence of tNASP are transported into nuclei.
D. Under the energy deficient conditions of 4°C there is no H1t nuclear transport.
E. Under the energy deficient conditions of no ATP there is no H1t nuclear transport.
F. H1t histones at 37°C in the presence of the nuclear localization signal deletion mutant (NASP-ΔNLS) are not transported into nuclei.
G. Somatic H1s at 37°C are not detected within the nuclei.
H. Somatic H1s at 37°C in the presence of tNASP are transported into nuclei.
Table 1. Translocation of linker histones into HeLa cell nuclei

| Treatment                        | Average # of cell counted +/- S.D. (n=3) | Average # of fluorescent nuclei (n=3) | Percent fluorescent nuclei | Significance           |
|----------------------------------|------------------------------------------|---------------------------------------|---------------------------|------------------------|
| H1t                              | 247 ± 61.3                               | 4.3 ± 1.5                             | 2                         |                        |
| H1t + cytosol                    | 232 ± 28.5                               | 128.7 ± 14.8                          | 55                        | P<.005                 |
| H1t + NASP                       | 213 ± 37.5                               | 137.7 ± 21.5                          | 65                        | P<.009                 |
| H1t + NASP @ 4°C                 | 250 ± 37.8                               | 8.3 ± 7.1                             | 3                         | P<.006 vs. NASP        |
| H1t + NASP without ATP           | 243 ± 39.2                               | 26.3 ± 10.6                           | 11                        | P<.005 vs. NASP        |
| H1t + NASP-ΔNLS                  | 220 ± 18.7                               | 6.3 ± 1.5                             | 3                         | P<.009 vs. NASP        |
| H1                               | 221 ± 1.7                                | 3 ± 2.7                               | 1                         |                        |
| H1 + cytosol                     | 258 ± 32.9                               | 134.3 ± 44.5                          | 52                        | P<.04                 |
| H1 + NASP                        | 217 ± 29.5                               | 148 ± 27.7                            | 68                        | P<.02                 |
| H1 + NASP @ 4°C                  | 259 ± 57.1                               | 3 ± 0                                 | 1                         | P<.012 vs. NASP        |
| H1 + NASP without ATP            | 217 ± 32.6                               | 15 ± 2.7                              | 7                         | P<.014 vs. NASP        |
| H1 + NASP-ΔNLS                   | 241 ± 62.2                               | 10 ± 6.3                              | 4                         | P<.011 vs. NASP        |
