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The Y-located gonadoblastoma gene *TSPY* amplifies its own expression through a positive feedback loop in prostate cancer cells

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ABSTRACT

The testis-specific protein Y-encoded (*TSPY*) is a repetitive gene located on the gonadoblastoma region of the Y chromosome, and has been considered to be the putative gene for this oncogenic locus on the male-only chromosome. It is expressed in spermatogonial cells and spermatocytes in normal human testis, but abundantly in gonadoblastoma, testicular germ cell tumors and a variety of somatic cancers, including melanoma, hepatocellular carcinoma and prostate cancer. Various studies suggest that TSPY accelerates cell proliferation and growth, and promotes tumorigenesis. In this report, we show that TSPY could bind directly to the chromatin/DNA at exon 1 of its own gene, and greatly enhance the transcriptional activities of the endogenous gene in the LNCaP prostate cancer cells. Domain mapping analyses of TSPY have localized the critical and sufficient domain to the SET/NAP-domain. These results suggest that TSPY could efficiently amplify its expression and oncogenic functions through a positive feedback loop, and contribute to the overall tumorigenic processes when it is expressed in various human cancers.

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1. Introduction

Numerous studies have suggested that genes on the male specific Y-chromosome play a wide spectrum of functions, beside sex determination, and mutations of which contribute a variety of male-enhanced/restricted diseases, such as azoospermia [1], hearing loss [2], heart and cardiovascular diseases [3], testicular germ cell tumors [4], and selected somatic cancers [5–7]. Among the male-specific genes, the testis-specific protein Y-encoded (*TSPY*) is a tandem repeated (>40 times) ampliconic gene, and is the putative gene for the gonadoblastoma locus on the Y chromosome (*GBY*), the only oncogenic locus on the male-specific chromosome. TSPY is expressed in spermatogonial cells and spermatocytes of normal testis, and has been postulated to serve important functions in spermatogonial renewal and meiotic division in male germ cells [8–11]. TSPY is also abundantly expressed in gonadoblastoma and various types of germ cell tumor including seminoma and carcinoma-in-situ/intratubular germ cell neoplasia unclassified (CIS/ITGCNU) [12–14], and various somatic cancer types, including melanoma, liver cancer, and prostate cancer [15–19]. TSPY harbors a domain, called SET/NAP domain, identified among the SET oncoprotein and nucleosome assembly proteins (NAPs). It interacts with type B cyclins via the SET/NAP domain and increases the cyclin-B/CDK1 kinase activity [20], and with the translation elongation factor EEF1A and enhances protein synthesis [21]. Hence, TSPY could exert various cell cycle and growth regulation functions and exacerbates cancer development through these pathways [22–24]. Indeed over-expression of TSPY accelerates cell proliferation and tumor growth in xenograft model mice, and up-regulate various oncogenes and pro-growth genes [25]. Ectopic expression of TSPY in ovaries of transgenic mice results in gonadoblastoma-like structures [26], suggesting that *TSPY* is a male specific and proto-oncogene on the Y chromosome, and its expression could indicate a male-specific or biased oncogenic action(s) in cancer development and progression.

In the present study, we demonstrate that TSPY protein is capable of binding to the chromatin/DNA regions of the endogenous *TSPY* gene, and increasing the its own expression in a prostate cancer cell line, LNCaP. We have mapped the effective domain to the SET/NAP-domain, and the cis-element to exon 1 of the *TSPY* gene, suggesting a potential positive feedback loop for TSPY amplification of its own expression.

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2. Materials and methods

2.1. Plasmid constructs

The coding sequence of full-length human TSPY (variant-1; NM_003308.3) [21] was tagged with a FLAG epitope, and inserted in the mammalian expression vector, pIRES2-EGFP plasmid (Clontech Laboratories Inc., Mountain View, CA), which is capable of expressing the FLAG-TSPY and the EGFP reporter in the same transcript. A nuclear localization signal (NLS) was inserted between FLAG and TSPY coding sequences to target FLAG-TSPY to nucleus [21]. Firefly luciferase reporter pGL2-based or pGL4.27-based vectors containing different fragments of human TSPY gene promoter and/or exon 1 regions were constructed: pGL2-TSPY[P], pGL2-TSPY[P+E], pGL4.27-TSPY[+47/+146], pGL4.27- [+97/+296], pGL4.27-TSPY[+97/+196], pGL4.27-TSPY[+197/+296], pGL4.27-TSPY[+247/+396], pGL4.27-TSPY[+347/+446], pGL4.27-TSPY[+397/+496]. The fragments were generated by PCR using pTSPY12.5 [9] as a template and primers indicated in Table S1. The PCR products were ligated into pGEM-T easy cloning vector (Promega, Madison, WI), and subcloned into the multi-cloning site of pGL2-basic vector (Promega) or pGL4.27 vector (Promega). pGL4.27 vector harbors a minimal promoter (minP) between multi-cloning site and luciferase gene. All recombinant constructs were confirmed by DNA sequencing and analysis.

2.2. Cell lines

LNCaP cells were grown in RPMI-1640 with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were transfected with pIRES2-GEFP or pIRES2-EGFP-FLAG-TSPY using FuGENE6 (Roche, Indianapolis, IN). Forty-eight hr after transfection, cells were supplied with fresh medium containing 450 μg/mL G418. Seventeen days after G418 selection, the EGFP-positive cell populations were purified by flow cytometry using the FACSAria cell sorter (BD Biosciences) and maintained in culture media containing 450 μg/mL G418.

2.3. Immunofluorescence

Cells were seeded at 25% confluency onto chamber slides (Lab-Tek II CC2, Thermo Scientific Nunc) and cultured for 2 days. Immunofluorescence was performed as described previously [21] with anti-GFP rabbit (Abcam) and anti-TSPY mouse monoclonal antibodies [27].

2.4. Western blot analysis

Western blot analysis was performed as described previously [21]. The membranes were probed with anti-FLAG rabbit IgG (Sigma–Aldrich), anti-TSPY rabbit serum [21], anti-GFP rabbit IgG (Abcam), and anti-β-actin mouse IgG (Sigma–Aldrich).

2.5. Gene expression analysis

Total RNAs were isolated by using RNeasy mini kit (QIAGEN), and analyzed by qRT-PCR in triplicates, using GoTaq qPCR Master Mix (Promega) and MyiQ real-time PCR detection system (BioRad, Hercules, CA). The primer sequences are described in Table S1.

2.6. Chromatin immunoprecipitation (ChIP)

LNCaP-TSPY cells were processed for chromatin immunoprecipitation (ChIP) assay using EZ magna ChIP kit (Millipore), using anti-FLAG mouse IgG (clone M2, Sigma–Aldrich), anti-RNA polymerase II mouse IgG (Millipore) or control mouse IgG (1.5 μg/reaction), according to the manufacturer's protocol. ChIP DNA samples were analyzed by PCR (ChIP-PCR) or quantitative PCR (ChIP-qPCR) using primers for TSPY gene (Table S1).

2.7. Reporter assay

LNCaP cells were seeded at 1×10^5 cells per well on 24 well plates 24 h prior to transfection. The cells were co-transfected with 0.1 μg/well pGL2-base or pGL4.27-base luciferase vector constructs, 0.4 ng/well phRL-CMV Renilla luciferase and 0.1–0.2 μg/well p3xFLAG-TSPY or p3xFLAG-CMV7, using X-tremeGENE9 (Roche). Forty-eight hours later, cells were harvested in 80 μl of reporter lysis buffer (Promega) and stored as complete cell lysis at −80 °C. The firefly and *Renilla* luciferase activities were analyzed by using the Dual luciferase reporter assay system (Promega) and TD-20/20 luminometer (Turner Biosystems, Sunnyvale, CA) according to the manufacturer's instruction.

3. Results

3.1. Stable expression of a TSPY transgene up-regulates the expression of its endogenous gene in the LNCaP cells

TSPY is abundantly expressed in clinical prostate cancer, particularly in specimens of high stages of malignancy [18,19]. Various studies suggest that over-expression of TSPY could have a positive effect(s) on oncogenic processes [25]. To evaluate its potential oncogenic role in prostate cancer, we have stably transfected a FLAG-tagged TSPY recombinant construct in the pIRES2-EGFP vector to the prostatic LNCaP cells, using neomycin selection and flow cytometry strategy. Immunofluorescence and Western blot analysis showed that LNCaP cells harboring the FLAG-tagged TSPY (LNCaP-TSPY) expressed the TSPY abundantly while those harboring the pIRES2-EGFP vector alone (LNCaP-EGFP) did not (Fig. 1A and B). TSPY was distributed evenly between the nuclei and cytoplasm of the transfected cells, similar to those observed in human testicular germ cells [8,21], seminoma [21] and neurons [28]. The prostatic cancer cell line, LNCaP, harbors the human Y chromosome, and expresses the endogenous TSPY in relatively low levels, as compared to the exogenously transfected TSPY transgene, and is only detectable at RNA level but not at protein level (Fig. 1B).

To distinguish the expression of the transfected and endogenous *TSPY* genes (Fig. 1C), specific primers were designed and used in quantitative RT-PCR analysis. Our results showed that the endogenous *TSPY* gene was significantly up regulated (>2 fold) in the LNCaP-TSPY cells (Fig. 1D). On the other hand, no significant change was observed in the expression level of the prostate specific antigen (*PSA*) in the same cells (Fig. 1D). These results suggested that TSPY could selectively enhance the expression of its own endogenous *TSPY* gene in a positive feedback loop.

3.2. FLAG-TSPY is specifically bound to the exon 1 region of the endogenous TSPY gene in LNCaP cells

The enhancement of its own endogenous gene expression by a transfected TSPY transgene is an interesting observation. Previously we had explored the potential targets for TSPY bindings to genes affected by its ectopic expression in a testicular embryonal cell line, NT2/D1, using the ChIP-Chip strategy (Kido and Lau, unpolished results). Among the 5 genes bound by the TSPY protein on the Y chromosome, two were endogenous *TSPY* genes, i.e. *TSPY1* and *TSPY2*. The TSPY binding was specifically localized at the transcription start site and exon 1 region (Fig. S1). To explore if TSPY was capable of binding to the same region of the endogenous gene

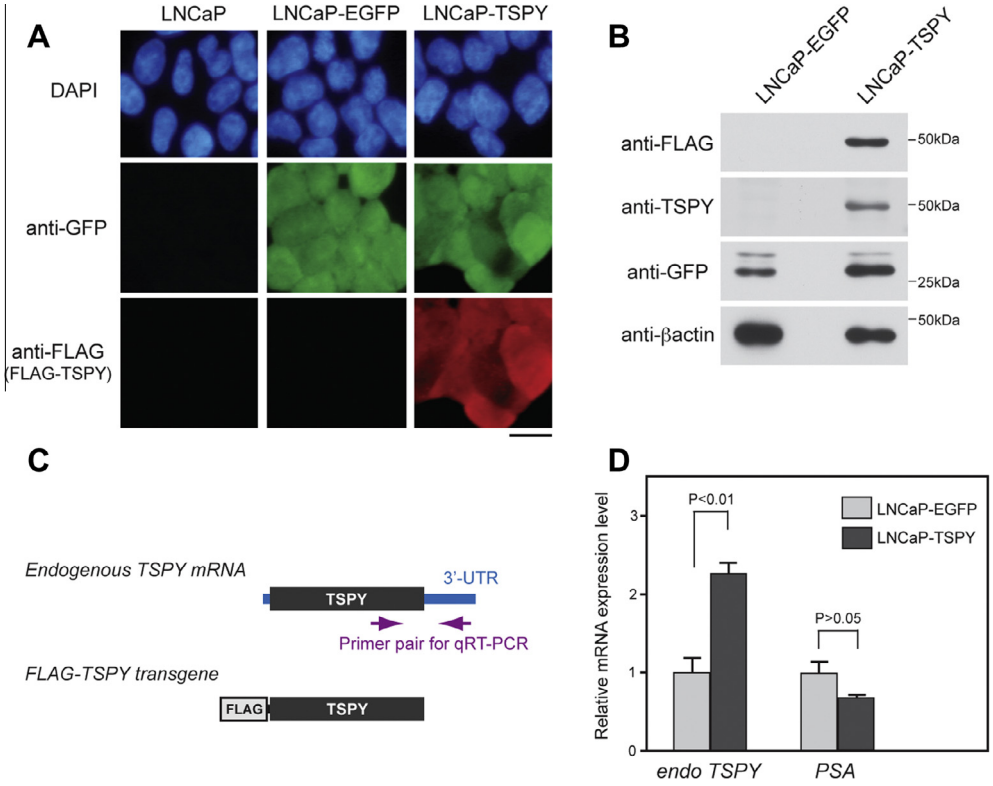

Fig. 1. Characterization of the LNCaP cells stably expressing EGFP (LNCaP-EGFP), or EGFP and FLAG-tagged TSPY (LNCaP-TSPY). (A) Immunofluorescence of parental LNCaP cells, LNCaP cells stably expressing EGFP alone (LNCaP-EGFP), or EGFP and FLAG-TSPY (LNCaP-TSPY) by using anti-GFP (green) and anti-FLAG (red) antibodies. Nuclear DNA was visualized by DAPI staining (blue). Note that FLAG-TSPY was localized in both nucleus and cytoplasm. Scale bar = 25 μm. (B) Western-blot analysis of the FLAG-TSPY and EGFP expression in LNCaP-EGFP and LNCaP-TSPY cells. The blots were probed with specific antibodies, as indicated. (C) Schematic illustration of endogenous TSPY transcript and FLAG-TSPY expression construct. Arrows indicate the positions of primers for qRT-PCR analysis. (D) qRT-PCR analysis of the expression levels of endogenous *TSPY* (*endo TSPY*) and prostate-specific antigen (*PSA*) in the LNCaP-EGFP and LNCaP-TSPY cells. Data were normalized against GAPDH expression level in each sample. Bars: means ± SE of 3 independent experiments. The expression level of endogenous *TSPY* was significantly higher in LNCaP-TSPY cells ($P < 0.01$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in LNCaP cells, chromatin immunoprecipitation (ChIP) was performed with an anti-FLAG antibody on LNCaP-TSPY cell lysate, and analyzed with specific primers flanking the transcription start site, i.e. −164 to +271 position of the *TSPY* endogenous gene. Our results showed that the TSPY protein was associated with DNA/chromatin at the transcription start site and exon 1 region of the *TSPY* transcriptional unit, which was also bound by RNA polymerase II (Fig. 2). These observations suggest that TSPY bound to the exon 1 region and its flanking sequences of the endogenous genes in LNCaP cells, similar to those observed in the embryonal carcinoma NT2/D1 cells.

3.3. TSPY stimulation of its endogenous gene expression is depending on the exon 1 sequences

Based on these findings, we had focused our efforts on the transcription start site and exon 1 as a candidate region responsible for TSPY stimulation of its own expression. Initially, the promoter (TSPY[P]) and the promoter plus exon 1 (TSPY[P+E]) were inserted immediately upstream of the promoter-less luciferase reporter construct, pGL2 (Fig. 3A). These luciferase constructs were transfected transiently to the LNCaP cells with increasing amounts of co-transfected FLAG-TSPY expression construct. Our results showed that the TSPY product was capable of stimulating the luciferase activities with the pGL2-TSPY[P+E] construct, but not those of pGL2-TSPY[P] promoter alone or vector alone (Fig. 3B). These initial observations showed that sequence at exon 1 could harbor important cis-element(s) for TSPY stimulation of its own

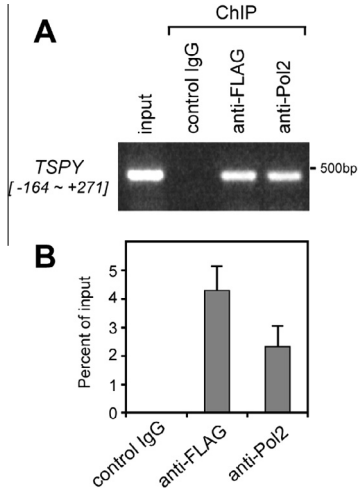

Fig. 2. Chromatin immunoprecipitation (ChIP) analysis on the binding of FLAG-TSPY protein onto the endogenous *TSPY* gene. (A) LNCaP-TSPY cells were subjected to ChIP-PCR analysis using anti-FLAG, anti-RNA polymerase II and control IgG. Immunoprecipitated DNA was analyzed by PCR with primer pairs for the genomic region around the transcription start sites (TSS) of endogenous *TSPY* gene. (B) Immunoprecipitated DNA samples in A was subjected to qPCR analysis. The enrichment of a chromatin fraction is defined as a ratio of precipitated DNA over input DNA. qPCR reactions were carried out in triplicates using fixed amounts of template DNA from each fraction. Bars: means ± SE of 3 independent experiments.

expression at a dosage dependent manner. To further narrow down the specific region in exon 1 responsible for such increases in gene

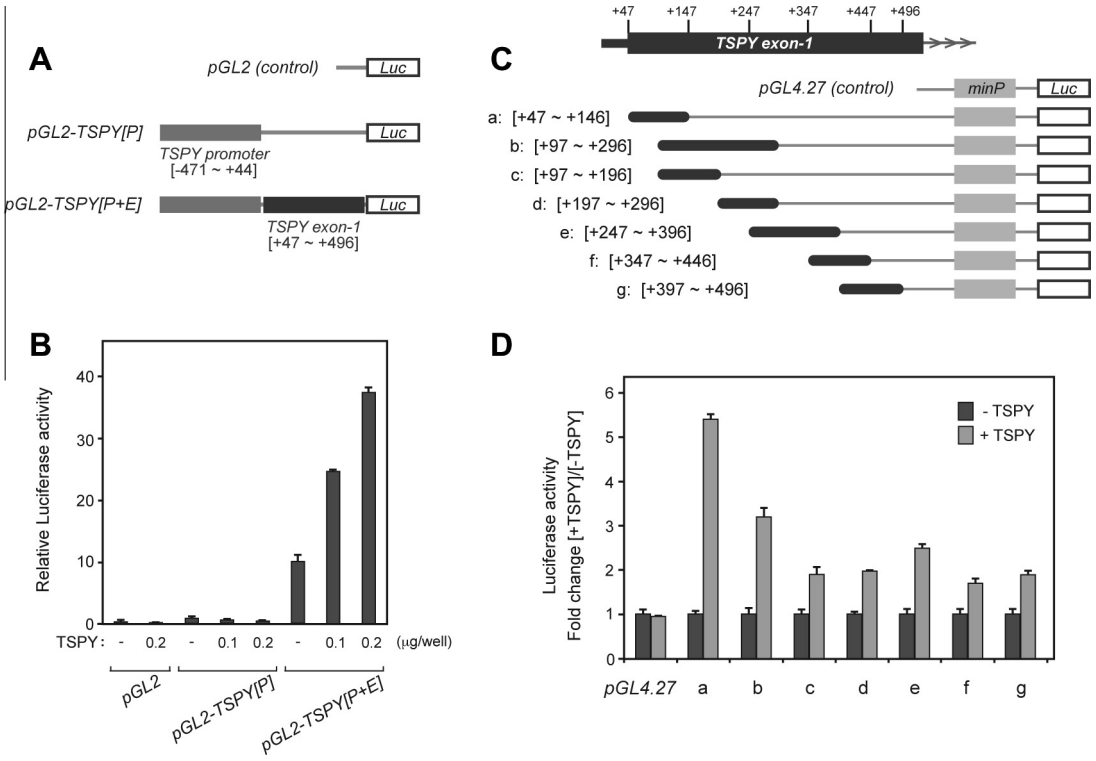

Fig. 3. Luciferase reporter assay of TSPY-dependent *TSPY* gene activation. (A) Schematic representation of luciferase reporter constructs to investigate the effects of TSPY on the transcription activity of its own gene. (B) Reporter constructs shown in A were co-transfected with various amount of FLAG-TSPY expression vector or control plasmid (p3xFLAG-CMV7) into LNCaP cells. Luciferase activities were measured 48 h after transfection. The data represent the means ± SE of 4 individual experiments. Note that co-transfection with FLAG-TSPY expression vector significantly enhanced the activity of the reporter construct containing the exon 1 sequences of *TSPY* gene. (C) Schematic representation of reporter constructs for deletion mapping of the TSPY-dependent enhancing element within exon 1. Regions indicated by bold lines (a-g) were inserted immediately upstream of the minimal promoter (minP) of pGL4.27 luciferase reporter plasmid. Positions relative to the transcription initiation site (TSS) are indicated. (D) Reporter constructs shown in C were co-transfected into LNCaP cells with 0.2 μg/well of FLAG-TSPY expression vector (+TSPY) or control plasmid (-TSPY). Luciferase activities were measured 48 h after transfection. The data represent the means ± SE of 4 individual experiments. Region a [+47 to +146] provided the strongest enhancing effect.

expression, a series of luciferase constructs with various deletion segments (Fig. 3C) from exon 1 were constructed and analyzed similarly with and without FLAG-TSPY expression vector. Our results showed that the most significant region was located to [+47 to +146] within exon 1 sequences, which showed >5 fold increase in TSPY stimulation while other selected regions within exon 1 exerted either weak or minimal TSPY enhancing effects in this reporter assay (Fig. 3C and D).

3.4. The SET/NAP-domain is essential for TSPY-dependent gene expression enhancement

The *TSPY* transcriptional units are capable of synthesizing various natural and alternatively spliced transcripts, as compared to the predominant full-length (variant 1) transcript encoding a protein with 308 amino acids [14,18]. To determine which domain of TSPY protein was essential for binding to exon 1 cis-elements and stimulating its endogenous gene expression, variant gene constructs encoding for different isoforms of TSPY transcripts were generated and analyzed with the reporter assay system. The variant H71 encodes a protein with an internal truncation of 87 amino acids [residue #30-116 inclusively] but still harbors an intact SET/NAP-domain, while variant H109 harbor an internal truncation of 139 amino acids [residue #30-168 inclusively] and a partial SET/NAP-domain (Fig. 4A). Immunofluorescence analysis of transfected LNCaP cells showed that all FLAG-tagged TSPY variants, full-length, H71 and H109, were localized in both nucleus and cytoplasm evenly, whereas variant H109 displayed a strong punctuated cytosolic pattern in some cells (Fig. 4B). The luciferase reporter pGL2-TSPY[P+E] harboring both *TSPY* promoter and exon 1 sequence and PGL2 luciferase vector alone (Fig. 3A) were used in co-transfection assays with expression constructs with full-length, variant H71, or variant H109 TSPY coding sequences. Our results showed that only the full-length and H71 variant TSPY proteins were capable of stimulating the pGL2-TSPY[P+E] reporter activity, while variant H109 protein did not show any stimulation (Fig. 4C). These results suggest that the intact SET/NAP-domain is essential for TSPY-dependent auto-stimulation of gene expression.

To further explore the importance of SET/NAP domain for TSPY gene stimulation, we performed a series of reporter assays in which the nuclear localized TSPY and TSPY [151-308] that contains an intact SET/NAP-domain alone were analyzed with the co-transfection of the pGL2-TSPY[P+E] reporter. By targeting the expression of TSPY variants to nucleus, cytoplasmic effect of TSPY could be minimized. Our results showed that both nuclear localized full-length TSPY and TSPY[151-308] stimulated the pGL2-TSPY[P+E] reporter activities as well as native TSPY (Fig. 4D), suggesting that SET/NAP domain itself is sufficient to stimulate the TSPY-dependent auto-stimulation of gene expression.

4. Discussion

TSPY is the putative gene for the *GBY* oncogenic locus on the human Y chromosome. It encodes a protein of 308 amino acids, which harbors a domain, termed SET/NAP domain, conserved among the SET oncoprotein and the nucleosome assembly proteins (NAPs). This group of SET/NAP domain-containing proteins has been designated as the TSPY/NAP/SET (TNS) family, whose members

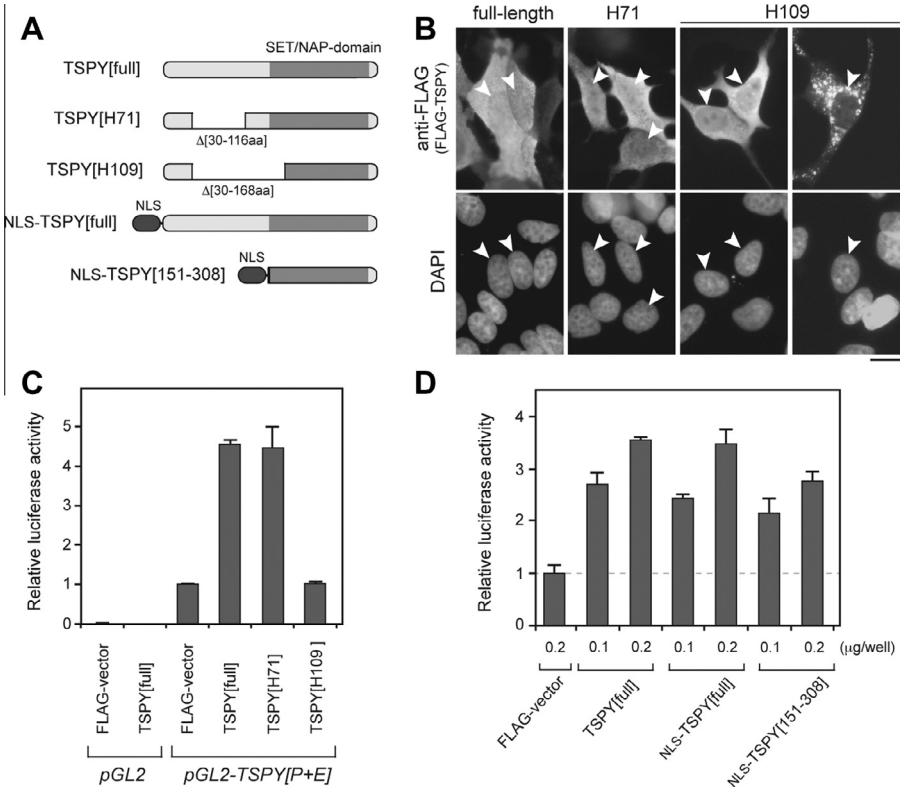

Fig. 4. Intact SET/NAP-domain is essential for the TSPY-dependent *TSPY* gene activation. (A) Schematic representation of the TSPY variants, examined in the present study. Variants H71 and H109 lack [30-116 amino acids] and [30-168 amino acids], respectively. (B) Immunofluorescence of LNCaP cells transiently transfected with the FLAG-TSPY variant expression vector, detected by anti-FLAG antibody (white in dark field). DNA was visualized by DAPI staining. Arrows indicate the nuclei of transfected cells. Scale bar = 10 μm. (C) The expression vectors of FLAG-TSPY variants were co-transfected with pGL2-TSPY[P+E] reporter plasmid or pGL2-basic plasmid into LNCaP cells, and analyzed as before. The data represent the means ± SE of 4 individual experiments. (D) Luciferase reporter assay investigating the effect of nuclear localized TSPY (NLS-TSPY[full]) and TSPY[151-308] (NLS-TSPY[151-308]) variants on pGL2-TSPY[P+E] reporter activity. The data represent the means ± SE of 4 individual experiments.

participate in numerous physiological functions, including cell cycle regulation, signaling transduction, and cell differentiation [29–33]. SET and NAP1 proteins interact with numerous cytoplasmic and nuclear target proteins, and play multiple functions such as histone chaperone, nucleosome assembly, chromatin modification, transcription regulation and other cellular functions [31,34–37].

TSPY is primarily expressed in normal testicular spermatogonial cells and spermatocytes and is postulated to serve important functions during male meiotic division [8,27]. Significantly, it is ectopically expressed in gonadoblastoma, testicular germ cell tumors, and various somatic cancers, including prostate cancer, melanoma and liver cancer [12–16,18,19]. TSPY interacts with the mitotic cyclin B and enhances the kinase activities of a cyclinB-CDK1 complex, which in turn accelerates a G2/M transition and cell proliferation [20]. Such abbreviation of the G2/M stage could potentially compromise the checkpoints at this critical stage of the cell cycle and promotes genomic instability through various mutational consequences, such as chromosome nondisjunction and other mitotic dysfunctions [38,39]. It also interacts with the translation elongation factor EEF1A, and promotes protein synthetic activities [21]. Indeed, forced expression of a TSPY transgene in HeLa and NIH3T3 cells increases cell proliferation and promotes tumorigenicity in athymic mice [25]. Hence, up-regulation of TSPY is closely associated with oncogenic activities. Since *TSPY* gene is located on the Y chromosome, such ectopic expression of a proto-oncogene suggests that TSPY could exert a male-specific tumorigenic function(s) on the initiation, progression and treatment responses of somatic cancers. In the present study, we demonstrated that forced TSPY expression in the LNCaP cells resulted in an increase in the expression on the endogenous *TSPY* gene, suggesting a potential positive feedback loop for this male-specific proto-oncogene, thereby amplification of its oncogenic functions in tumorigenesis. This finding provides new insight into the mechanism of TSPY in exacerbation of cancer progression in men.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.083.

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