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Cleavage of TFIIA by Taspase1 Activates TRF2-Specified Mammalian Male Germ Cell Programs

Toshinao Oyama[1,8], **Satoru Sasagawa**[5,8], **Shugaku Takeda**[1], **Rex A. Hess**[6], **Paul M. Lieberman**[7], **Emily H. Cheng**[1,2,4], and **James J. Hsieh**[1,3,4,*]

[1]Human Oncology & Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York, NY10065, USA

[2]Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY10065, USA

[3]Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY10065, USA

[4]Department of Medicine, Weill Medical College of Cornell University, New York, NY10021, USA

[5]Department of Biology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, JP

[6]Veterinary Biosciences, College of Veterinary Medicine, University of Illinois at Urbana-Champaign, Urbana, IL61802, USA

[7]The Wistar Institute, Pennsylvania, PA19104, USA

SUMMARY

The evolution of tissue-specific general transcription factors (GTF), such as testis-specific TBP-related factor 2 (TRF2), enables the spatiotemporal expression of highly specialized genetic programs. Taspase1 is a protease that cleaves nuclear factors MLL1, MLL2, TFIIAα-β, and ALFα-β (TFIIAτ). Here we demonstrate that Taspase1-mediated processing of TFIIAα-β drives mammalian spermatogenesis. Both *Taspase1−/−* and non-cleavable *TFIIAα-βnc/nc* testes release immature germ cells with impaired transcription of *Transition proteins (Tnp)* and *Protamines (Prm)*, exhibiting chromatin compaction defects, recapitulating those observed with *TRF2−/−* testes. Although the unprocessed TFIIA still complexes with TRF2, this complex is impaired in targeting and thus activating *Tnp1* and *Prm1* promoters. Current study presents a paradigm in which a protease (Taspase1) cleaves a ubiquitously expressed GTF (TFIIA) to enable tissue-specific (testis) transcription, meeting the demand for sophisticated regulation of distinct subsets of genes in higher organisms.

INTRODUCTION

RNA transcription is a highly orchestrated biochemical process that decodes genetic information encrypted in the DNA sequence, initiating the gene expression profile unique to

*Correspondence: hsiehj@mskcc.org.
[8]These authors contributed equally to this work.

SUPPLEMENTAL INFORMATION
Supplemental Information includes 4 figures and 1 table.

individual cells (Roeder, 1998). In principle, the rate-limiting step is the functional assembly of a transcription preinitiation complex (PIC) at the start site of RNA synthesis, which is influenced by DNA-sequence specific transcription factors and chromatin modifying/remodeling machineries (Hernandez, 1993; Hochheimer and Tjian, 2003; Li et al., 2007; Narlikar et al., 2002; Orphanides and Reinberg, 2002; Roeder, 1998; Veenstra and Wolffe, 2001). PIC, consisting of RNA polymerase (RNA Pol) and general transcription factors (GTFs; TFIIA, -B, -D, -E, -F, and -H), nucleates at promoters of individual genes and integrates signals to generate unique expression (Hahn, 2004). Unlike bacteria and archaea that utilize only one RNA Pol and one GTF σ, eukaryotes have evolved with three RNA polymerases (RNA Pol I–III) and distinct sets of GTFs to produce different classes of RNA.

Positioned at the center of eukaryotic GTFs is the TATA-box binding protein (TBP). TBP is the central constituent of SL1, TFIID, and TFIIIB complexes that locate promoters and recruit RNA Pol I, II and III, respectively (Hernandez, 1993; Hochheimer and Tjian, 2003). Unlike simple eukaryotes which contain only TBP, metazoans are evolved with additional TBP variants, namely TBP related factor 1–3 (TRF1-3) to accommodate the need for complex gene regulation (Reina and Hernandez, 2007). Although genetic studies have highlighted the critical role of TRFs in targeting large subsets of genes for critical developmental processes, the detailed mechanisms remain largely undetermined. TRF1, an insect-specific TBP that is enriched in the CNS and gonads during early embryonic development, regulates transcription from both Pol II and III promoters (Hansen et al., 1997; Takada et al., 2000). TRF2 (also known as TLF, TLP and TRP) (Dantonel et al., 1999; Moore et al., 1999; Ohbayashi et al., 1999; Rabenstein et al., 1999), present in all metazoans, is required for the embryonic development of *C. elegans*, *Xenopus laevis* and zebrafish, and spermatogenesis of mammals (Reina and Hernandez, 2007). TRF3 (TBP2), a vertebrate-specific TBP, is most closely related to TBP and is required for the embryogenesis of zebrafish and *Xenopus* (Reina and Hernandez, 2007).

Site-specific proteolysis regulates critical aspects of biology, such as the activation of blood coagulation factors for hemostasis, the activation of caspases for cell death execution, the cleavage of Notch intracellular domain for cell fate determination, the release of SREBP for cholesterol homeostasis, and the maturation of HCF and MLL1 for cell cycle progression (Capotosti et al., 2011; Julien and Herr, 2003; Takeda et al., 2006). Taspase1 was purified as the protease which cleaves MLL1 for proper *Hox* gene expression (Hsieh et al., 2003a; Hsieh et al., 2003b). *Taspase1* encodes a highly conserved 50kD α-β proenzyme which undergoes intramolecular autoproteolysis to produce the mature α28/β22 heterodimeric enzyme that displays an overall α/β/β/α structure (Hsieh et al., 2003a; Khan et al., 2005). Taspase1 is the only protease within a family of enzymes possessing an Asparaginase_2 homology domain (Hsieh et al., 2003a). Other members present in prokaryotes and eukaryotes include the amidohydrolases: L-asparaginase and glycosylasparaginase. L-asparaginase is involved in asparagine metabolism and glycosylasparaginase participates in the ordered breakdown of N-linked glycoproteins. Taspase1-mediated cleavage follows distinct aspartate residues of conserved IXQL(V)D/G motifs (Chen et al., 2012), suggesting that Taspase1 evolved from hydrolyzing asparagines and glycosylasparagines to cleaving polypeptides after aspartates. We named it Taspase1 (threonine aspartase) according to its mechanism of action and the discovery of which founded a class of endopeptidases that utilizes threonine to cleave polypeptide substrates after P1 aspartates (Hsieh et al., 2003a). All of the confirmed Taspase1 substrates are nuclear factors that play important roles in gene regulation. *Bona fide* Taspase1 substrates include MLL1, MLL2, TFIIAα-β, and *Drosophila* HCF (dHCF) (Capotosti et al., 2007b; Takeda et al., 2006; Zhou et al., 2006). Remarkably, like the protease itself, all Taspase1 substrates were translated as α-β precursors that undergo proteolysis to form mature α/β heterodimers. Subsequent studies identified additional *bona fide* Taspase1 substrates, including MLL2, TFIIAα-β, ALFα-β

(TFIIA like factor, also known as TFIIAτ) and *Drosophila* HCF (dHCF) (Capotosti et al., 2007a; Takeda et al., 2006; Zhou et al., 2006). Interestingly, all Taspase1 substrates are nuclear factors that participate in transcription regulation.

The recognition of core promoters by TBP (TATA box binding protein) is further enforced by the association of TFIIA and TFIIB (Bleichenbacher et al., 2003; Jacobson and Tjian, 1996). Unlike the yeast TFIIA that comprises only large and small subunits, TFIIA in arthropods and mammals comprises three polypeptides (α, β, and γ) (DeJong and Roeder, 1993; Ma et al., 1993; Ozer et al., 1994; Ranish and Hahn, 1991; Yokomori et al., 1993). Taspase1 cleaves precursor TFIIAα-β to generate processed α and β subunits that interact with the THIIAγ subunit to form TFIIAα/β/γ. In higher organisms, TBP related factors (TRFs) have evolved to target large subsets of tissue/organ-specific promoters (Bartfai et al., 2004; Persengiev et al., 2003; Rabenstein et al., 1999; Takada et al., 2000). As TFIIAα/β/γ forms complexes with TBP and TRFs, TFIIAα-β and its respective cleavage could potentially regulate gene expression through various forms of TFIIA-TBP/TRF-DNA complexes.

Our initial characterization of *Taspase1*−/− mice demonstrated the role of MLL1 and MLL2 proteolysis in regulating *Hox* and *Cyclin* genes and thus orchestrating segmental body plan and cell cycle, respectively (Takeda et al., 2006). Here, we report that proteolytic processing of precursor TFIIAα-β by Taspase1 is required for the execution of testis-specific transcription program.

RESULTS

Taspase1 is Required for Mammalian Spermatogenesis

The majority of *Taspase1*−/− mice died at postnatal day 1 due to feeding defects (Takeda et al., 2006). Upon examinations of survived *Taspase1*−/− mice, we noticed that all *Taspase1*−/− males are infertile (n > 10), correlating with the abundant expression of *Taspase1* in testis. *Taspase1*−/− males exhibited normal copulating behavior and analysis of their blood did not suggest dysregulation of key pituitary gonadotropic hormones and testicular androgens as the cause (Figure S1A). Dissection of the urogenital system identified small testes (~50% reduction in weight compared to *wild-type* littermates) as the only macroscopic defect (Figure 1A). Histological inspection of *Taspase1*−/− epididymis where mature sperms are normally stored demonstrated the lack of mature spermatozoa (Figure 1B). Of note, *Taspase1*−/− females were fertile.

Mammalian male germ cell development (spermatogenesis) occurs in successive waves of mitotic, meiotic, and postmeiotic (spermiogenesis) phases (Russell et al., 1990). The spermatogenic cycle of mice can be subdivided into 12 stages with each stage consisting of distinct arrays of male germ cells (Figure S1B). Spermatogonia, the male germ stem cells, amplify by mitosis to self-renew and differentiate into spermatocytes that enter meiosis, generating post-meiotic round, haploid spermatids. These round spermatids progress through a 16-step maturation program called “spermiogenesis” by which they morphologically and structurally transform through elongating spermatid steps (8–16) into fully differentiated spermatozoa with mature acrosomes, condensed nuclei, and mitochondria-enriched flagella (Russell et al., 1990).

Of note, the spermatogenic cycle of adult mice is asynchronous. Microscopic examinations of adult *Taspase1*−/− testes uncovered a ~33% reduction in the diameter of seminiferous tubules (Figure 1C). These *Taspase1*−/− tubules consisted of fewer germ cell layers with severely diminished number of elongating spermatids (Figure 1D and S1C). Detailed microscopic analyses demonstrated that most of the post-meiotic *Taspase1*−/− round

spermatids fail to advance beyond step 11 spermiogenesis, resulting in a marked decrease of elongating spermatids (8.8%) in comparison with *wild-type* littermate testes (32.9%) (Figure 1E). The exhaustion of germ cell layers along with formation of empty vacuoles raised a possibility of increased cell death and/or impaired proliferation in addition to the observed differentiation block. Accordingly, TUNEL and anti-PCNA IHC assays were performed. We observed an increase in apoptosis but no impairment in proliferation (Figure 1F and 1G).

Taspase1 Deficiency Disrupts the First Wave of Spermatogenesis

To further pinpoint the postmeiotic spermiogenic defects incurred by the loss of *Taspase1*, we examined juvenile mouse testes (week 1 to 5) where the first wave of developing germ cells progress synchronously through spermatogenesis with the sequential appearance of specific mitotic, meiotic, and post-meiotic cells (Figure 2). The lack of maturing, elongating spermatids was evident at week 4, which was followed by tubular degeneration and formation of empty vacuoles at week 5. At the end of the first postnatal spermatogenic cycle, there was a lack of discernible spermatozoa in *Taspase1*$^{-/-}$ testes (Figure 2). Collectively, our data demonstrated an essential role of Taspase1 in the proper development of mammalian male germ cells, especially at the late spermiogenic steps.

Taspase1 Regulates Transcription of Spermiogenic Genes and thereby Controls Chromatin Compaction

During spermiogenesis, nuclear histones are replaced by Transition proteins (Tnp1 and Tnp2)–a transitional step before the ultimate compaction of haploid DNA around Protamines (Prm1 and Prm2) (Eddy, 1998; Kleene, 1989). Consequently mice deficient for *Tnps* or *Prms* are infertile due to impaired chromatin condensation in elongating spermatids (Cho et al., 2001; Zhao et al., 2004). As elongating spermatids are transcriptionally silent, nearly all transcription during spermiogenesis takes place in round spermatids (Kleene et al., 1984; Monesi et al., 1978), e.g. *Tnps*, *Prms and GAPDHs* (Kleene, 1989; Welch et al., 1992). When testis-specific transcription programs were evaluated by quantitative RT-PCR in 3 week-old *Taspase1*$^{-/-}$ juvenile testes, we discovered profoundly compromised expression of *Tnps* and *Prms* but not ubiquitously expressed housekeeping genes (Figure 3A). When 3 month-old *Taspase1*$^{-/-}$ adult testes were assayed, severe transcription defects of spermiogenic genes, *Tnps* and *Prms*, to ~26–40% of *wild-type* testes were observed (Figure 3B). We examined whether the reduced expression of *Tnps* and *Prms* is simply due to the lack of 1N spermatids. Consistent with our morphological analysis (Figure 1E), FACS (fluorescence-activated cell sorting) of *Taspase1*$^{-/-}$ adult testes demonstrated reduced numbers of 1N spermatids to ~2/3 of *wild-type* ones (Figure S2). Accordingly, spermatid specific genes *Brd2* and *Brd3* (Okada et al., 2007; Shang et al., 2004) were proportionally reduced to ~70% of adult *Taspase1*$^{-/-}$ testes (Figure 3B). Altogether, data indicated the presence of specific transcription defects of *Tnps* and *Prms* when Taspase1 is lost.

In contrast to the abundance of mature spermatozoa in *wild-type* corpus epididymis, only scattered immature germ cells were detected in *Taspase1*$^{-/-}$ corpus epididymis (Figure 3C). We examined whether germ cells recovered from the *Taspase1*$^{-/-}$ corpus epididymis display DNA compaction defects with Acridine Orange. Spermatozoa with complete chromatin condensation stain green with Acridine Orange, whereas elongating spermatids with incomplete condensation stain both green and red (Kosower et al., 1992; Okada et al., 2007). The majority of *Taspase1*$^{-/-}$ germ cells were stained positive for both green and red (Figure 3D), indicating chromatin condensation defects. Altogether, our molecular data demonstrate that Taspase1 is required for proper transcription of *Tnps* and *Prms*, key spermiogenic genes.

Taspase1-mediated Cleavage of MLL1 and MLL2 is not Required for Spermatogenesis

Taspase1 orchestrates gene expression through cleaving precursor nuclear factors. The most characterized Taspase1 substrates are MLL1 and MLL2 (Liu et al., 2007; Takeda et al., 2006). Among MLL family proteins (MLL1-5), only MLL1 and MLL2 are Taspase1 substrates (Hsieh et al., 2003a; Liu et al., 2009). MLL3 and MLL4 (also known as ALR) are distantly related to MLL1 and MLL2. MLL5 is an even further distant member (Figure 4A). The Taspase1-mediated cleavage of MLL1 and MLL2 renders their full HMT activity required for proper cell fate decision and cell cycle progression (Takeda et al., 2006). To determine whether non-cleavage of MLL1 and MLL2 accounts for the germ cell defects observed in *Taspase1*−/− testes, we examined *MLL1nc/nc;2nc/nc* mice carrying homozygous non-cleavable alleles of *MLL1* and *MLL2* at endogenous loci (Takeda et al., 2006). *MLL1nc/nc;2nc/nc* males were fertile and displayed no gross or microscopic abnormalities in their testes and epididymides (Figure 4B and 4C). Therefore, Taspase1 likely activates spermiogenic programs by cleaving other nuclear transcription factors.

The Expression of Taspase1 and its Substrates TFIIA and ALF in Mouse Testis

Besides MLL1 and MLL2, *bona fide* Taspase1 substrates in mammals are TFIIAα-β and ALFα-β (TFIIA like factor) (Figure 5C, S3A and S3B) (Chen et al., 2012; Zhou et al., 2006). Precursors TFIIAα-β and ALFα-β undergo Taspase1-mediated proteolysis, generating mature TFIIAα/β and ALFα/β heterodimers (Figure S3B). To investigate whether Taspase1 regulates spermatogenesis through cleaving TFIIAα-β and/or ALFα-β, we determined the expression of Taspase1, TFIIA and ALF in juvenile and adult testes by IHC and immunoblots (Figure 5A and B).

In juvenile testes, Taspase1 as well as TFIIAα/β was weakly expressed before week 2, became abundant from week 3 when spermiogenesis begins, and maintained high expression through maturation (Figure 5A and B). By contrast, ALFα/β was barely detectable at week 3 and became actively expressed from week 4 (Figure 5A and 5B). Of note, a well-demarcated spatiotemporal expression of TFIIAα/β and ALFα/β in both juvenile and adult testes was observed (Figure 5A), implicating sequential employment of these factors during male germ cell transcription. In human testis, ALFα/β is also translated late during spermatogenesis and was proposed to involve the final morphogenesis of elongating spermatids (Huang et al., 2006). In the absence of Taspase1, spermatogenic defects manifested as early as postnatal week 3 when spermiogenesis begins (Figure 2). These defects correlate well with the expression pattern of Taspase1 and TFIIAα-β, suggesting an important role of the Taspase1-TFIIA proteolytic axis in mammalian male germ cell differentiation. As most *Taspase1*−/− spermatids failed to progress through elongating spermatids, we could only detect a weak expression of ALFα-β in *Taspase1*−/− adult testes (Figure S3C). Accordingly, the role of ALFα-β cleavage in spermatogenesis cannot be adequately assessed in these animals. Collectively, our phenotypic, genetic, and gene expression analyses support a working model in which Taspase1 cleaves TFIIAα-β to activate spermiogenic genes.

Processed TFIIα/β is Required for the Functional Assembly of TRF2-Specific Transcription Machinery on Spermiogenic Promoters

TFIIA has long been considered as a GTF that complexes with TBP to initiate RNA polymerase II controlled transcription. In metazoans, three variants of TBP have been discovered: TRF1 (insect-specific), TRF2 (metazoan-specific), and TRF3 (TBP2; vertebrate-specific) (Bartfai et al., 2004; Persengiev et al., 2003; Rabenstein et al., 1999; Takada et al., 2000). Among these TBPs, the testis-enriched TRF2 plays an essential role in mammalian spermatogenesis (Zhang et al., 2001). The fact that TFIIA complexes with TRF2 to regulate transcription raised a testable hypothesis that Taspase1-mediated cleavage of TFIIAα-β is required to assemble an active TFIIA-TRF2 complex on the promoters of

Tnps and *Prms*, which is highly supported by the near identical spermatogenic defects shared by *Taspase1−/−* and *TRF2−/−* mice (Zhang et al., 2001).

We first examined whether the loss of Taspase1 affected the expression of TRF2. There is comparable expression of TRF2 in *Taspase1−/−* and *wild-type* adult testes (Figure 5C). We also determined whether non-cleavage of TFIIAα-β disrupts its interaction with TRF2 in testicular cells. Interestingly, both processed TFIIAα/β and precursor TFIIAα-β interacted with TRF2 (Figure 5C), excluding a possibility that Taspase1-mediated cleavage of TFIIAα-β is a prerequisite for its interaction with TRF2. Interestingly, precursor TFIIAα-β accumulated abundantly in *Taspase1−/−* testes (Figure 5C), which is consistent with a prior report showing that processed TFIIAα/β is more susceptible to proteasome-mediated degradation (Hoiby et al., 2004). We subsequently assessed whether Taspase1 deficiency affects targeting of the TFIIA-TRF2 complex to *Tnp1* and *Prm1* promoters in testes. Chromatin Immunoprecipitation (ChIP) assays using *Taspase1−/−* testes demonstrated decreased binding of TFIIA and TRF2 to *Tnp1* and *Prm1* promoters (Figure 5D). By contrast, the targeting of TFIIA to *β-Actin* promoter was minimally affected (Figure 5D), likely reflecting the known regulation of *β-Actin* by TBP, instead of TRF2, in testes (Zhang et al., 2001). Furthermore, ChIP assays did not detect decreased H3K4me3 on *Tnp1* and *Prm1* promoters in *Taspase1−/−* testes (Figure 5D), suggesting that H3K4me3 renders a permissive state allowing for transcription activation and predates the assembly of basal transcription machinery in this context.

Non-cleavable *TFIIAα-βnc/nc* Testes Exhibit the Same Morphological and Molecular Defects as *Taspase1−/−* Testes

Our study thus far demonstrates a critical role of Taspase1 in orchestrating mammalian male germ cell transcription program through proteolytic cleavage of TFIIAα-β in testis. Such a working model is quite intriguing in that both Taspase1 and TFIIAα-β are nearly ubiquitously expressed. To unequivocally address the significance of Taspase1-mediated TFIIAα-β cleavage in spermatogenesis, we created a mouse model in which the endogenous D/G cleavage residues of TFIIAα-β are replaced with non-cleavable (nc) A/A (Figure 6A). The non-cleavage of TFIIAα-β in *TFIIAα-βnc/nc* mice was confirmed by immunoblots using testicular extracts (Figure 6B). Of note, the non-cleavable TFIIAα-β is more abundant than processed TFIIAα/β (Figure 6B), mirroring what was observed in *Taspase1−/−* testis (Figure 5C).

Similar to *Taspase1−/−* mice, *TFIIAα-β*nc/nc mice were born at 21% but most died within a day after birth, likely due to feeding defects, accounting for 4.1% of total animals when genotyped at 3 weeks of age (Table S1). Remarkably, testis weight of *TFIIAα-βnc/nc* adult mice was at ~51% of *wild-type* littermates (Figure 6C). The majority (8 of 13) of *TFIIAα-βnc/nc* adult male mice were infertile, and the remaining 5 were able to impregnate female mice despite small testes. Of note, like *Taspase1−/−* female mice, most *TFIIAα-β*nc/nc females were fertile. Histological examinations of infertile *TFIIAα-βnc/nc* testes revealed near identical microscopic defects as depicted in *Taspase1−/−* testes, including the absence of mature spermatozoa in epididymis (Figure 6D), the reduced germ cell layers of seminiferous tubules, the lack of elongating spermatids (Figure 6E), and the increased apoptosis (Figure 6F). Anti-PCNA staining did not detect impaired proliferation (Figure 6G). Detailed examinations of *TFIIAα-βnc/nc* adult testes also revealed a differentiation block with markedly reduced elongating spermatids (7.6%) (Figure 6H and 1E). Collectively, our data demonstrated that both infertile *TFIIAα-βnc/nc* and *Taspase1−/−* testes shared the same macro- and microscopic abnormalities as *TRF2−/−* testes (Zhang et al., 2001).

TFIIAα-βnc/nc Testes Exhibit the Same Molecular Defects as *Taspase1 −/−* Testes

To investigate whether infertile *TFIIAα-βnc/nc* adult testes exhibit the same molecular defects as *Taspase1−/−* adult testes, quantitative RT-PCR, Acridine Orange staining, and ChIP assays were performed. Remarkably, we discovered (1) low expression of *Tnps* and *Prms* (Figure 7A); (2) defects in sperm DNA compaction (Figure 7B); and (3) impaired *Tnp* and *Prm* promoters targeting by non-cleavable TFIIAα-β and TRF2 (Figure 7C). Furthermore, levels of *Tnps* and *Prms* in 3 and 4 week-old *TFIIAα-βnc/nc* juvenile testes (Figure 7A) were as low as observed in *Taspase1−/−* juvenile testes (Figure 3A). Interestingly, in *TFIIAα-βnc/nc* adult testes, lower *Tnps* and *Prms* expression correlates with infertility (Figure 7A), dichotomized at ~50% expression of *wild-type* testes (Figure S4), implicating stochastic compensatory mechanisms at play such as ALFα/β.

In summary, two independent genetic mouse models and associated biochemical analyses are in full support of a model in which Taspase1 proteolytically activates TFIIAα-β and thus enables assembly of an active TFIIA-TRF2 complex on the promoters of *Tnp1* and *Prm1* in spermatids to execute spermiogenic programs (Figure 7D).

DISCUSSION

Taspase1 is a highly conserved protease that orchestrates a plethora of genetic programs through cleaving nuclear transcription regulators, MLL1, MLL2, TFIIA, ALF, and dHCF (Capotosti et al., 2007b; Liu et al., 2007; Takeda et al., 2006). Our prior studies demonstrated that Taspase1 activates the full histone methyl transferase activity of MLL1 and MLL2 to regulate the expression of *Hox* and *Cyclin* genes for cell fate determination and cell cycle progression, respectively (Chen et al., 2010; Takeda et al., 2006). Here, we demonstrate that Taspase1 regulates testis-specific genetic programs through cleaving TFIIA to enable the functional assembly of TFIIA-TRF2 complexes on spermiogenic promoters. Hence, Taspase1 is uniquely empowered to organize complex genetic programs through (1) cleaving MLL1 and MLL2 to fine-tune chromatin status and (2) processing TFIIA to initiate the assembly of tissue-specific transcription machinery.

The differentiation of spermatozoa from spermatogonia is a marvel of gene regulation, which has evolved in part by employing testis-specific transcription regulators such as TRF2, TAF4b, CREM, ERM, Plzf and JHDM2A (Buaas et al., 2004; Cooke and Saunders, 2002; Costoya et al., 2004; Falender et al., 2005; Okada et al., 2007). The coexistence of general and testis-specific transcription machineries within the same cell poses a unique challenge for balancing and bridging these respective networks of gene expression. Our study suggests that Taspase1-mediated proteolytic cleavage of TFIIAα-β could serve to interconnect and fine-tune the general and tissue-specific transcription. In somatic cells, precursor TFIIAα-β binds TBP and is functionally active in transcription (Zhou et al., 2006). Therefore, in this context the cleavage of TFIIAα-β may serve to fine-tune the rate of transcription through regulating its stability, as processed TFIIAα/β is known to be more susceptible to degradation (Hoiby et al., 2004). On the contrary, the non-cleavage of TFIIAα-β in testes compromises targeting of the testis-specific TFIIA-TRF2 transcription complex to spermiogenic promoters. Hence, Taspase1-mediated cleavage of TFIIAα-β constitutes a critical step in regulating TRF2-target genes in mammalian testes.

TBP consists of an N-terminal species-specific domain and a ~180 aa C-terminal conserved domain (CCD) that binds DNA and complexes with TFIIA and TFIIB. All TRFs contain CCD which encodes the bipartite repeat saddle domain. TRF2, the metazoan-specific TBP variant, is distantly related to TBP and shares ~40% sequence identity in the CCD (Moore et al., 1999; Ohbayashi et al., 1999). Detailed sequence analysis reveals that key amino acids crucial for the interaction of TBP with the minor groove of TATA box have diverged in

TRF2, which is consistent with the inability of TRF2 to target canonical TATA boxes (Hochheimer and Tjian, 2003). The notion that TRF2 might bind promoters with a completely different specificity is supported by genetic studies on *TRF2* deficient animals: deficiency of TBP or TRF2 apparently incurs deregulation of distinct genetic networks and results in distinct phenotypes (Jacobi et al., 2007; Martianov et al., 2002; Zhang et al., 2001). Although TRF2 has a diverged DNA recognition domain, its stirrup motifs that mediate TFIIA and TFIIB binding are conserved, reflecting known preserved interactions (Hochheimer and Tjian, 2003). The TFIIA in flies and mammals comprises three polypeptides encoded by *TFIIAα-β* and *TFIIA*γ (Hoiby et al., 2007). The posttranslational proteolytic processing of precursor TFIIAα-β is executed by Taspase1–a critical step required for the activation of TRF2-but not TBP-target genes in the mammalian testis. Hence, the cleavage of TFIIA might function to balance these two co-existing transcription machineries.

The discovery and study of Taspase1 provides an opportunity to investigate how a site-specific protease interconnects diverse genetic networks through respective proteolyses. Non-cleavage of Taspase1 substrates such as MLLs and TFIIA apparently produces *hypomorphic* alleles instead of *null* alleles. Therefore, Taspase1 deficiency offers an unprecedented opportunity to uncover signaling pathways controlled by these essential genes of which complete deletion often results in early embryonic lethality. Indeed, Taspase1 not only regulates cell cycle, cell fate, and male germ cell development, but also controls fetal liver hematopoietic stem cell and craniofacial development (Unpublished data). The vertebrate-specific TBP variant TRF3 is essential for embryonic development in *Xenopus* and zebrafish (Bartfai et al., 2004; Hart et al., 2007; Jallow et al., 2004). As TRF3 also binds TFIIA (Bartfai et al., 2004), it is conceivable that Taspase1 may organize additional genetic programs through modulating the TFIIA-TRF3 complex. Hence, the Taspase1-TFIIA axis likely helps orchestrate the functional assembly of diverse tissue-specific transcription machinery required for the development of higher organisms. Further characterizations of *Taspase1−/−* and noncleavable Taspase1 substrates animals would further broaden our knowledge concerning the elaborate gene regulation circuitry in mammals.

EXPERIMENTAL PROCEDURES

Targeting Construct and Generation of Non-cleavable TFIIAα-β Mice

The murine *TFIIAα-β* genomic locus sequences were obtained from the emsembl.org database. The targeting construct has a *loxP* site upstream to exon 6 and a *loxP*-flanked *pPGKneo* cassette that was inserted into the intron between exons 7 and 8. The D/G residues of the Taspase1 cleavage site on exon 6 were mutated to A/A by site-directed mutagenesis. Successfully targeted RW4 embryonic stem cells (129SvJ) were determined by PCR analyses, followed by a transient transfection with CMV-Cre to generate *TFIIAα-βnc/+* ES clones with deletion of *pPGKneo*. ES cells with correct targeting were injected into C57Bl/6 blastocysts to generate *TFIIAα-βnc/+*mice that were used for intercross to generate *TFIIAα-βnc/nc* mice.

Mice

Taspase1−/− and *MLL1nc/nc;2nc/nc* mice were previously reported (Takeda et al., 2006). *Taspase1−/−* and *TFIIAα-βnc/nc* mice were maintained in mixed C57BI/6;129SvJ background to mitigate severe postnatal lethality associated with pure genetic background. All mouse experiments were conducted in accordance with institution guidelines.

Statistical Analysis

Statistical significance of differences between two groups was assessed by unpaired Student *t* test using the R software (http://www.r-project.org).

Quantitative RT-PCR

Q-RT-PCR analyses using SYBR Green Master Mix (Applied Biosystems) were performed as previously described (Hsieh et al., 2003a). 18S rRNA and GAPDH kits were purchased from Applied Biosystems. Sequences of oligonucleotides are listed in Supplementary Experimental Procedures.

Western Blot and Co-immunoprecipitation Assays

Testicular extracts were homogenized using polytron or FastPrep-24 (MP Biomedicals) and solubilized in NTTD buffer (150mM NaCl, 50mM TrisHCl pH8.0, 0.5% TritonX-100, 1mM DTT) supplemented with 1x protease and phosphatase inhibitors (Roche, Complete Protease Inhibitor; Calbiochem, Phosphatase inhibitor cocktails I & II). Co-IP and Western blot analyses were performed as previously described. Anti-TFIIA antibodies utilized in Co-IP and Western blot analyses were generated against the α subunit of mouse TFIIA (Covance). Anti-Taspase1 antibodies in Western blot analyses were generated against the β subunit of human Taspase1 (ProMab) (Chen et al., 2010). Western blot images were acquired with the LAS-3000 Imaging system (FujiFilm) and analyzed by ImageGuage software as previously described (Chen et al., 2010).

Chromatin Immunoprecipitation Assays (ChIP)

ChIP assays were performed as previously described with minor modifications (Takeda et al., 2006). Assays were performed using Magna ChIP G kit (Upstate) or performed using Dynabeads (Invitrogen). Analyses were performed on ABI 7200 or ViiA 7 systerm using gene-specific Taqman probes (IDT) as previously described (Takeda et al., 2006). Antibodies utilized include anti-TFIIA (Covance), anti-TRF2 (TLF) (Santa Cruz), and anti-H3 trimethyl K4 (abcam). Sequences of oligonucleotides and Taqman probes utilized are listed in Supplementary Experimental Procedures.

Hormone Assays

Blood was drawn from 3 month-old adult mice of the indicated genotypes and heparinized plasma was separated by centrifugation. Levels of the indicated hormones were determined by Center for Research in Reproduction Ligand Assay and Analysis Core at the University of Virginia.

Histology, Immunofluorescence, and TUNEL Assays

Testes and epididymides were dissected and fixed in Bouin's fixative and embedded in paraffin. Hematoxylin and Eosin stain was performed according to the standard protocol. For high resolution histology, testes were fixed with 1% glutaraldehyde, embedded in Technovit GMA7100 (EBScience), sectioned at 3 μm, and stained with PAS-Hematoxylin. For immunofluorescence assays, samples were sectioned at 8 μm using microtome (Leica) and incubated in 20mM of Tris-HCl (pH9.0) at 70°C overnight to retrieve antigens. Samples were treated with 1% blocking reagent (Roche) for 30 minutes and labeled with indicated primary antibody for 2hours at room temperature. Commercial antibodies utilized include anti-TFIIA (Santa Cruz), anti-ALF (Abcam), and anti-PCNA (Santa Cruz). Samples were subsequently stained with Alexa-conjugated secondary antibodies (Invitrogen). Nucleus was stained with Hoechst 33342 (Invitrogen). TUNEL assays were performed according to the manufacture's protocol (Roche). Acridine Orange staining was performed as previously

described (Okada et al., 2007) and counted the number of staining positive-cells by ImageJ software and ICTN plugin (Center for Bio-Image Informatics).

Sub-staging Analyses

The germ cells in 3 month-old seminiferous tubules of the indicated genotypes were counted and categorized into 4 groups based on their developmental status. Data were obtained from 9 tubules from 3 independent mice of the indicated genotype.

DNA ploidy analyses

Adult mice testes were minced with scissors and testicular cells were suspended in TNE buffer (0.01M Tris-HCl (pH 7.5), 0.15M NaCl, 0.001M EDTA) and dissociated with 55 μm nylon mesh. Testicular cells were stained with propidium iodide (Sigma-Aldrich) and analyzed by FACSCalibur (BD Biosciences).

In Vitro Cleavage Assays

35S-methionine-labeled in vitro transcribed/translated TFIIAα-β or ALF substrate was incubated with recombinant Taspase1 in cleavage buffer (100 mM HEPES [pH 7.9], 5 mM MgCl2, 20 mM KCl, 5 mM DTT, and 10% sucrose) for 30 minutes at 30°C. The respective proteolyses were analyzed by SDS-PAGE (Hsieh et al., 2003a).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- Taspase1 cleaves TFIIA to orchestrate mammalian male germ cell transcription programs
- TFIIA noncleavage (nc) results in sperm compaction defects due to low TNPs and PRMs
- Cleaved TFIIA complexes with TRF2 to form testis-specific transcription machinery
- *Taspase1*$^{-/-}$ and *TFIIA nc/nc* mice exhibit the same testicular defects as *TRF2*$^{-/-}$ mice

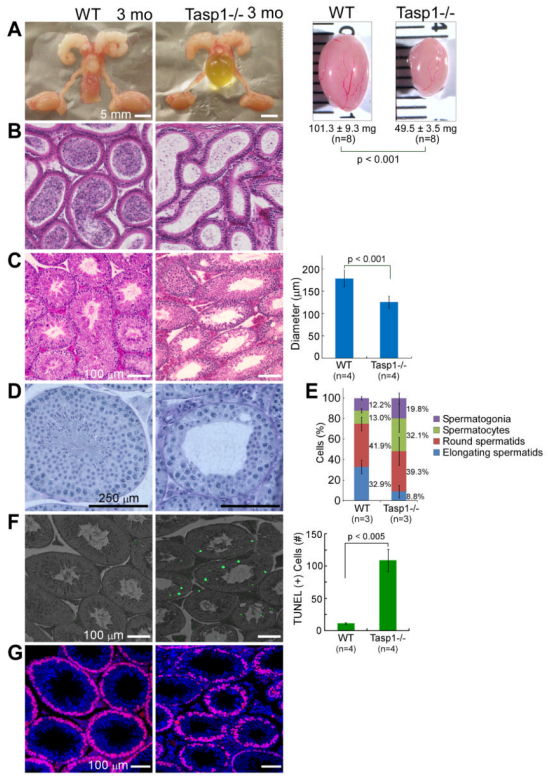

Figure 1. Taspase1 is Required for Mammalian Spermatogenesis

(**A**) Testes of *Taspase1*−/− male mice are smaller. The testis weight was presented as mean ± s.d. (n=8 per genotype).

(**B**) Hematoxylin & Eosin (H&E) stain of cross-sections of *Taspase1*−/− epididymides demonstrates the lack of mature sperms.

(**C**) H&E stain of cross-sectioned testes demonstrates the lack of mature spermatozoa and the reduction in diameter of seminiferous tubules of *Taspase1*−/− testes. The diameters of 200 seminiferous tubules were measured (n=4 per genotype). To obtain the real diameter of each tubule irrespective of sectioning angles, the shortest diameter of individual seminiferous tubules was used. Data presented are mean ± s.d.

(D) PAS-Hematoxylin staining of cross-sectioned adult testes. *Taspase1* deficiency resulted in fewer germ cell layers in adult seminiferous tubules

(**E**) Sub-staging of cells in adult seminiferous tubules. Adult *Taspase1*−/− testes contained fewer elongating spermatids than littermate *wild-type* testes (n=3 per genotype).

(**F**) Apoptotic cells were labeled by TUNEL assays and visualized as green signals by fluorescence microscopy. Signals were overlaid on phase contrast images. Bar graph represents TUNEL positive cells in 50 seminiferous tubules (n=4 per genotype). Data presented are mean ± s.d.

(G) Testicular cells in active DNA replication were labeled with anti-PCNA antibody (red) and visualized with DAPI staining (blue). All specimens were obtained from 3 month-old adult mice of the indicated genotypes.

See also Figure S1.

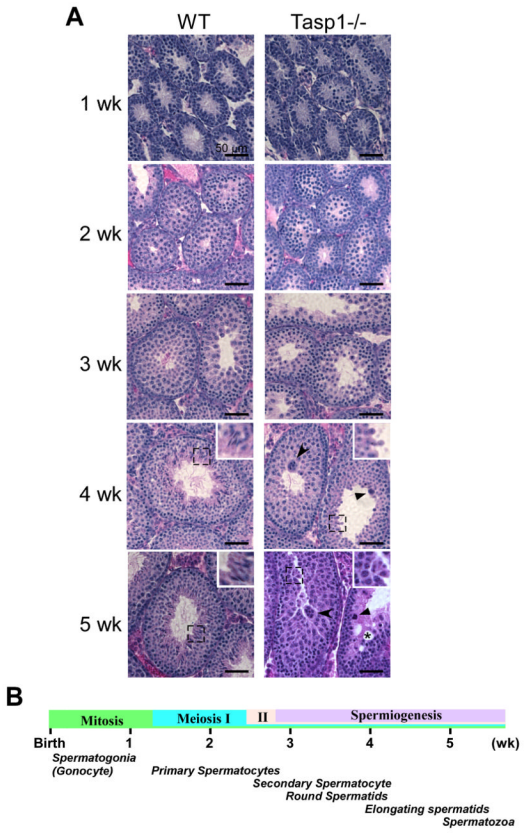

Figure 2. *Taspase1* Deficiency Disrupts the First Wave of Spermatogenesis in Juvenile Testes

(**A**) H&E sections of juvenile testes at the indicated ages are presented. Insets represent higher magnification images of the indicated squares. Solid triangles, arrow heads, and asterisks mark apoptotic cells, multinucleated giant cells, and empty vacuoles, respectively. In testis, multinucleated giant cells are commonly associated with increased apoptosis (Zhang et al., 2001).

(**B**) The diagram outlines the chronological order of the first wave of spermatogenesis in the mouse testis.

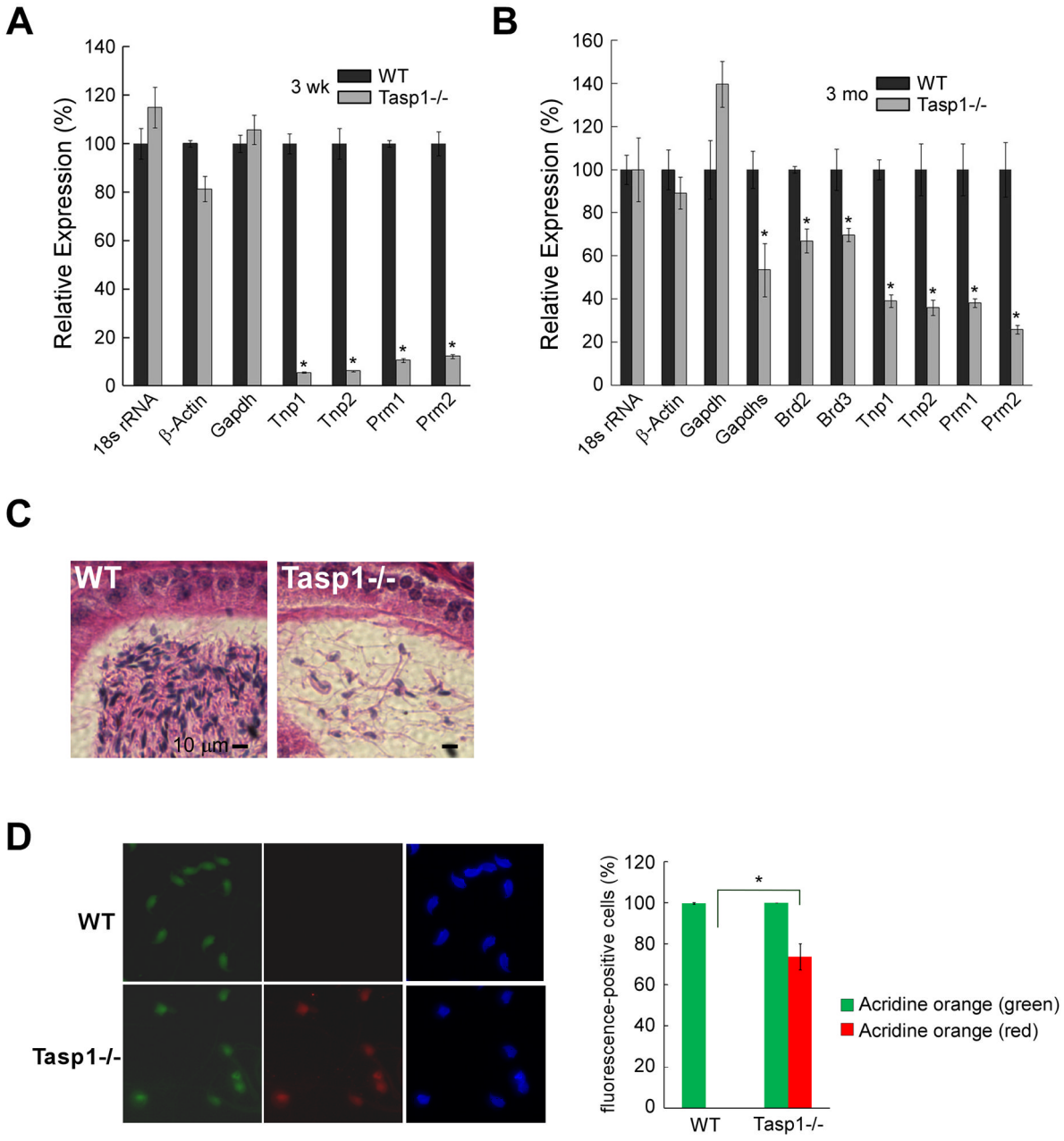

Figure 3. Taspase1 Activates Spermiogenic Genes and Regulates Chromatin Compaction

(**A** and **B**) Taspase1 deficiency disrupts the expression of spermiogenic genes. Quantitative RT-PCR analysis of expression of individual genes in 3 week-old (**A**) and 3 month-old testes (**B**) of the indicated genotypes. The transcript level of individual genes in *wild-type* (WT) testes was assigned as 100%. Data presented as mean ± s.d were obtained from at least three independent experiments.

(**C**) The H&E stain of cross-sections of 3 month-old corpus epididymides.

(**D**) Germ cells purified from corpus epididymides of the indicated genotypes were labeled with Acridine Orange and Hoechst. Upon Acridine Orange staining, spermatozoa with complete chromatin condensation are stained only positive for green, and germ cells with incomplete condensation are positive for both green and red. Blue staining represents

Hoechst. All specimens were obtained from 3 month-old adult mice of the indicated genotypes. The graph represents the percentage of the Acridine Orange positive cells. Data shown as mean ± s.d. were acquired from 5 representative areas (*$p < 0.001$).
See also Figure S2.

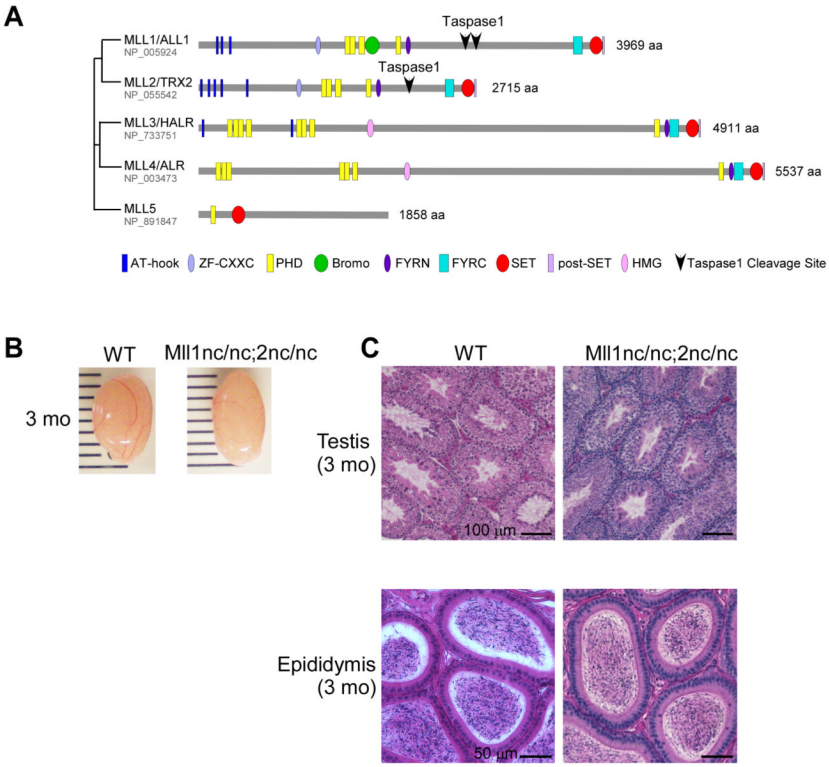

Figure 4. The Taspase1-mediated Cleavage of MLL1 and MLL2 is not Required for Spermatogenesis

(**A**) A diagram depicts the structural relationship among MLL family proteins (MLL1-5). Only MLL1 and 2 are Taspase1 substrates.

(**B**) There is no difference in the size of testes between *wild-type* and *MLL1nc/nc;2nc/nc* mice.

(**C**) H&E stain of testes and epididymides of the indicated genotypes. All specimens were from 3 month-old adult mice.

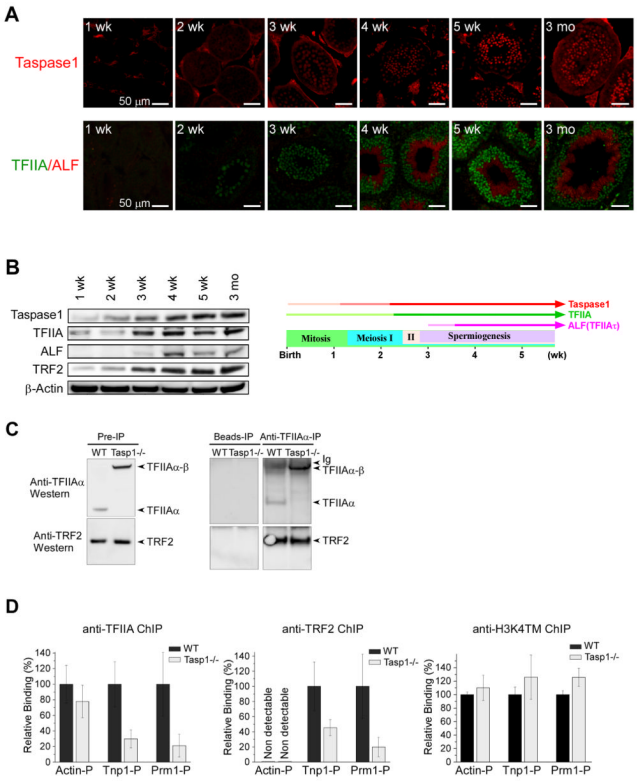

Figure 5. Taspase1-mediated Cleavage of TFIIAα-β Enables the Assembly of Active Testis-specific Transcription Machinery

(**A**) The expression of Taspase1 (red, upper figures), TFIIA (green, lower figures) and ALF (red, lower figures) during testicular development. Cross-sections of *wild-type* juvenile and adult testes of the indicated ages (week-old or month-old) are presented.

(**B**) The expression of Taspase1, TFIIA, ALF, TRF2, and β-Actin in *wild-type* testes at indicated age was determined by Western blot analyses. The diagram summarizes expression of Taspase1, TFIIA, and ALF based on immunofluorescence and Western blot analyses.

(**C**) Both cleaved and non-cleaved TFIIA interact with TRF2. Cellular extracts derived from 3 month-old adult testes of the indicated genotypes were subjected to co-immunoprecipitation assay using anti-TFIIA antibody. Precipitated TFIIA and co-precipitated TRF2 were visualized by Western blot analyses using the indicated antibodies.

(**D**) ChIP assays on the promoters of *β-Actin*, *Tnp1*, and *Prm1* were performed using the indicated antibodies (anti-TFIIA, anti-TRF2, anti-H3 trimethyl K4 (H3K4TM)) and analyzed by quantitative PCR. Data shown as mean ± s.d. were obtained from at least three independent experiments using 3 month-old adults testes of the indicated genotypes.

See also Figure S3.

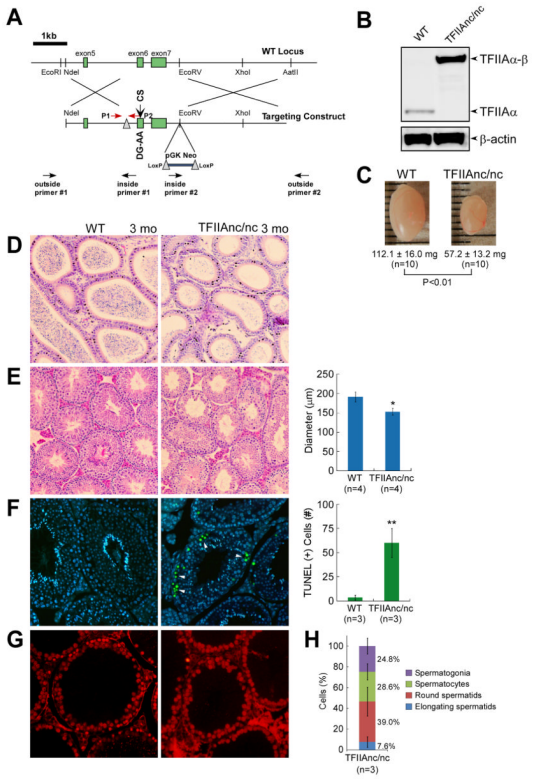

Figure 6. *TFIIAα-βnc/nc* Male Mice Exhibited the Same Testicular Phenotypes as *Taspase1−/−* Male Mice

(**A**) A schematic representation of the endogenous murine *TFIIAα-β* locus (top) and the targeting construct (bottom). solid triangles: loxP sites; CS: Taspase1 cleavage site; DG-AA: AA substitution of DG; arrows: PCR primers for genotyping.

(**B**) The absence of processed TFIIAα protein in *TFIIAα-βnc/nc* testes. Protein lysates of the indicated genotypes were extracted from 3 month-old adult mice testes and subjected to Western blot analysis using anti-TFIIAα antibody.

(**C**) Testes of *TFIIAα-βnc/nc* male mice are smaller than their *wild-type* littermates. Testis weight is presented as means ± s.d. (n=10 per genotype).

(**D** and **E**) H&E stain of cross-sectioned epididymides (**D**) and testis (**E**) from 3 month-old adult *TFIIAα-βnc/nc* mice demonstrates the lack of mature spermatozoa and the reduction in diameter of seminiferous tubules. The diameters of 200 seminiferous tubules were measured (n=4 per genotype, *P<0.001).

(**F**) Apoptotic cells, labeled by TUNEL assays and visualized as green signals by fluorescence microscopy, were increased in the *TFIIAα-βnc/nc* testis. DNA was stained by Hoechst (blue). Bar graph represents TUNEL positive cells in 50 seminiferous tubules (n=3 per genotype, **P<0.005).

(**G**) No decreased proliferation in spermatogonia of *TFIIAα-βnc/nc* testes. Testicular cells in active DNA replication were labeled with anti-PCNA antibody and visualized by fluorescence microscopy.

(H) The graph denotes percentages of distinct cell types in adult 3 month-old *TFIIAα-βnc/nc* seminiferous tubules. Data were acquired from 3 independent adult testes.

See also Table S1.

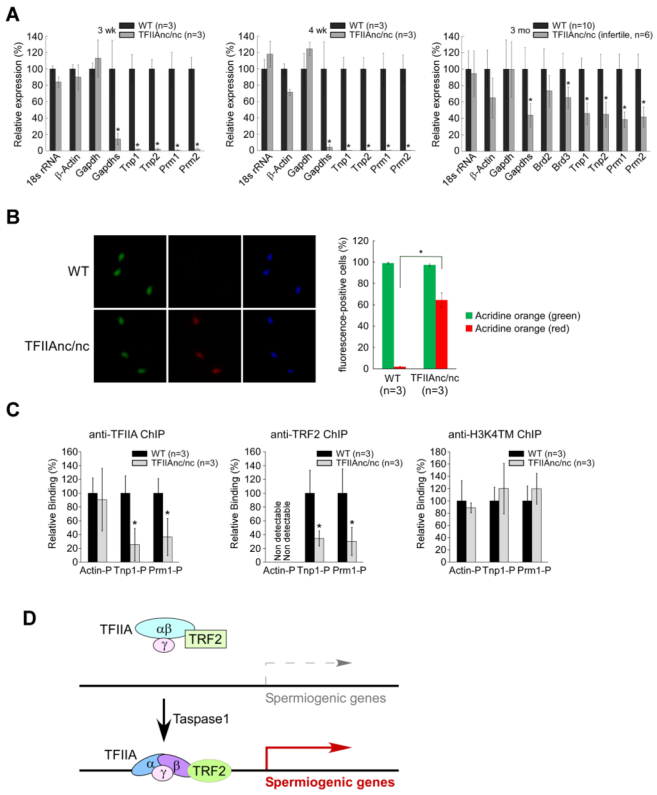

Figure 7. The Tapase1-mediated Cleavage of TFIIAα-β is Required for the Expression of Spermiogenic Genes in Juvenile and Adult Testes

(**A**) The expression of individual genes was determined by Q-RT-PCR in 3 week-old, 4 week-old, and 3 month-old testes. The transcript level in *wild-type* testes was assigned as 100%. Data presented as mean ± s.d. were obtained from at least three individual testes. *p < 0.001

(**B**) Germ cells purified from 3 month-old corpus epididymis of the indicated genotypes were labeled with Acridine Orange and Hoechst. The spermatozoa derived from *TFIIAα-βnc/nc* mice display incomplete chromatin condensation. The graph denotes the percentage of the Acridine Orange positive cells in 9 representative areas. Data were obtained from three independent experiments per genotype (*p < 0.001).

(**C**) ChIP assays demonstrate that Taspase1-mediated cleavage of TFIIAα-β is required for the assembly of active testis-specific transcription machinery. Data shown as mean ± s.d. were obtained from 3 independent experiments using 3 month-old testes of the indicated genotypes. *p < 0.05

(**D**) Model depicts how the Taspase1-TFIIA-TRF2 axis orchestrates mammalian spermatogenesis. TFIIA consists of three subunits: α and β subunits are derived from the post-translational cleavage of the gene product of *TFIIAα-β* by Taspase1, and the γ subunit is encoded by *TFIIAγ*.

See also Figure S4.