Targeted Disruption of Ing2 Results in Defective Spermatogenesis and Development of Soft-Tissue Sarcomas

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Abstract

ING2 (inhibitor of growth family, member 2) plays pivotal roles in the regulation of cellular senescence, apoptosis, DNA damage repair, gene transcription and chromatin modification [1,2]. Our previous in vitro studies on cellular senescence suggested that ING2 functionally interplays with the p53 tumor suppressor protein in two different manners: endogenous ING2 inhibits senescence and the transcriptional repression of ING2 by p53 abrogates this inhibition [3]; and overexpressed ING2 enhances p53 acetylation and stability to induce senescence [4,5]. ING2, as a subunit of the mSin3A-HDAC1 complex, specifically binds to tri-methylated lysine 4 of histone H3 (H3K4me3) to regulate chromatin modification and gene expression. ING2 also functionally interacts with the tumor suppressor protein p53 to regulate cellular senescence, apoptosis and DNA damage response in vitro, and is thus expected to modulate carcinogenesis and aging. Here we investigate the developmental and physiological functions of Ing2 through targeted germline disruption. Consistent with its abundant expression in mouse and human testes, male mice deficient for Ing2 showed abnormal spermatogenesis and were infertile. Numbers of mature sperm and sperm motility were significantly reduced in Ing2−/− mice (~2% of wild type, P<0.0001 and ~10% of wild type, P<0.0001, respectively). Their testes showed degeneration of seminiferous tubules, meiotic arrest before pachytene stage with incomplete meiotic recombination, induction of p53, and enhanced apoptosis. This phenotype was only partially abrogated by concomitant loss of p53 in the germline. The arrested spermatocytes in Ing2−/− testes were characterized by lack of specific HDAC1 accumulation and deregulated chromatin acetylation. The role of Ing2 in germ cell maturation may extend to human ING2 as well. Using publicly available gene expression datasets, low expression of ING2 was found in teratozoospermic sperm (>3-fold reduction) and in testes from patients with defective spermatogenesis (>7-fold reduction in Sertoli-cell only Syndrome). This study establishes ING2 as a novel regulator of spermatogenesis functioning through both p53- and chromatin-mediated mechanisms, suggests that an HDAC1/ING2/H3K4me3-regulated, stage-specific coordination of chromatin modifications is essential to normal spermatogenesis, and provides an animal model to study idiopathic and iatrogenic infertility in men. In addition, a bona fide tumor suppressive role of Ing2 is demonstrated by increased incidence of soft-tissue sarcomas in Ing2−/− mice.

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Introduction

ING2 [inhibitor of growth family, member 2] plays pivotal roles in the regulation of cellular senescence, apoptosis, DNA damage repair, gene transcription and chromatin modification [1,2]. Our previous in vitro studies on cellular senescence suggested that ING2 functionally interplays with the p53 tumor suppressor protein in two different manners: endogenous ING2 inhibits senescence and the transcriptional repression of ING2 by p53 abrogates this inhibition [3]; and overexpressed ING2 enhances p53 acetylation and stability to induce senescence [4,5]. ING2, as a subunit of the mSin3A-HDAC1 [histone deacetylase 1] complex, specifically binds to tri-methylated lysine 4 of histone H3 (H3K4me3) via its plant homeodomain (PHD) finger and regulates gene expression through chromatin modifications in response to DNA damage [6,7]. Although these findings imply that ING2 may contribute in vivo to p53-regulated processes, as well as developmental and homeostatic processes involving chromatin regulation, the in vivo physiological roles of ING2 have not been experimentally examined.

Spermatogenesis, a series of spermatogenic cell differentiation steps from spermatogonia to mature spermatozoa in the testes, is a
process tightly regulated by chromatin modifications [8,9]. Enzymes that modify histone methylation, including Meisetz (an H3K4 tri-methyltransferase) [10], G9a [a mono- and dimethyltransferase on lysine 9 of histone H3 (H3K9)] [11], and Swi39h1 and Swi39h2 (H3K9 tri-methyltransferases) [12], are essential for normal germ cell development in mice. The stage-specific acetylation profiles of several lysine residues on core histones, i.e., acetylation in spermatogonia and deacetylation during differentiation from leptotene to pachytene stages, are also critical to normal spermatogenesis in mice [8,13]. Consistently, HDAC inhibitors impaired male fertility in mice through loss of pachytene spermatocytes and increased apoptosis [14,15,16]. Such dynamic regulation of chromatin modifications during spermatogenesis is not limited to mice: the H3K4 methylation profiles during spermatogenesis were very similar between mice and non-human primates [17]. Misregulated histone acetylation is associated with defective spermatogenesis in humans [18], suggesting that chromatin-mediated regulation is a conserved mechanism from rodents to humans. However, no specific gene defect has been identified in humans as responsible for spermatogenic defect due to aberrant chromatin regulation [19].

In this study, our generation and characterization of Ing2-deficient mice reveal that Ing2 plays an essential in vivo role in mammalian spermatogenesis, which is attributed to its functional interaction with p53 and chromatin regulation. The relevance in humans is underscored by bioinformatics analysis revealing low ING2 expression in men with infertility and defective spermatogenesis. In addition, loss of Ing2 resulted in high incidence of soft-tissue sarcomas, particularly histiocytic sarcomas, demonstrating, for the first time, a tumor suppressor role for Ing2.

**Results**

**ING2 is expressed abundantly in mouse and human testes**

Quantitative RT-PCR (qRT-PCR) analysis of Ing2 mRNA expression in normal mouse tissues demonstrated a tissue-specific expression pattern, with testis showing the highest level of Ing2 (Fig. 1A), as has been reported in humans [20]. Immunohistochemical (IHC) staining of human testis sections showed that the cells in seminiferous tubules expressed higher levels of ING2 protein than the interstitial cells (Fig. S1). These data indicate that germ cells are the major source of ING2 expression in mouse and human testes.

**Generation of Ing2-deficient mice**

To examine the in vivo developmental and physiological roles of ING2, with particular interest to testicular development and function, mouse Ing2 gene was knocked out using a Cre-loxP recombination system (Fig. S2A). DNA genotyping (Fig. S2B), qRT-PCR analysis (Fig. S2C) and western blot analysis (Fig. S2D) confirmed the generation of mice with wild-type Ing2 (+/+), heterozygous for Ing2 knockout (+/−) and homozygous for Ing2 knockout (−/−). In crosses between heterozygous mice, the occurrences of Ing2+/+, Ing2+/− and Ing2−/− genotypes were 27% (154 out of 570), 56% (319 out of 570) and 17% (97 out of 570), respectively, showing a slight deviation from the expected Mendelian distribution and indicating that Ing2 deficiency may have a mild adverse effect on embryonic or prenatal development.

**Ing2-deficient males are infertile**

The postnatal growth of Ing2−/− mice was indistinguishable from that of their Ing2+/+ littermates. Ing2−/− mice had significantly smaller testes than those of Ing2+/+ mice throughout their life (Fig. 1C,D and Table 1, P<0.001), but did not show any gross abnormalities in other organs, including seminal vesicles, epididymides and vasa deferens (Fig. 1C, Table 1). The small-sized testes of Ing2−/− mice were not due to serum testosterone levels (Table 1).

When 8-week-old Ing2+/+, Ing2+/− and Ing2−/− mice were mated, Ing2−/− male mice were revealed to be infertile, while Ing2+/− and Ing2−/− female mice were fertile. The number of normal sperm found in semen from epididymis and vasa deferens in 8-week-old Ing2−/− males was only approximately 2% of that in age-matched Ing2+/+ control (Fig. 1E, F and Table 1, P<0.0001). The low sperm count in Ing2−/− mice became more severe with aging (Fig. 1F). In Ing2−/− mice, the sperm motility was also severely impaired (Table 1, P<0.0001) and almost all spermatozoa showed abnormal morphologies, such as round heads, short tails, large heads, multiple tails and tail coiling (Fig. 1G). Thus, male infertility in Ing2−/− mice is attributed to quantitative and qualitative defects in mature spermatozoa.

**Ing2−/− testes show degeneration of seminiferous tubules**

Histological analysis showed normal germ cell development in seminiferous tubules in Ing2+/+ testes (Fig. 2A,B). In contrast, Ing2−/− testes exhibited seminiferous tubule degeneration, germ cell under-population (Fig. 2C,D) with Leydig cell hyperplasia, apoptotic cells and multinucleated giant cells (Fig. 2E). Ing2−/− seminiferous tubules, spermatogonia are present in low numbers and in some tubules various developmental stages can be seen up to round spermatid. The major cell stage observed in these tubules is spermatocyte with large dense nuclei (Fig. 2D, 2B, black arrows). However, postmeiotic cell types such as round and elongated spermatids (Fig. 2B, white arrow) were scarcely observed in Ing2−/− testes (Fig. 2D), suggesting a spermatogenesis arrest at meiotic phase in most tubules. Observation of the epididymal wall also suggests that the cellular organization of this structure is also compromised in Ing2−/− mice. While mature spermatozoa were present in the epididymides of Ing2+/+ mice, hypospermia was observed in Ing2−/− (Fig. 2F).

Ing2+/+ and Ing2−/− testes were histologically examined throughout the mouse lifespan (Fig. S3). While Ing2+/+ seminiferous tubules developed normally and became completely populated with all stages of germ cells by 6 to 8 weeks, most Ing2−/− tubules remained devoid of germ cells and/or disorganized at the same age. Analysis at 6 through 24 months of age reveals that Ing2−/− tubules maintained normal germ cell development. Ing2−/− tubules, however, underwent progressive degeneration and germ cell depletion, eventually showing a Sertoli-cell-only pathology at 24 months. When the epididymis was examined at 24 months, mature spermatozoa were present in Ing2+/+, while the Ing2−/− epididymis displayed an aspermic phenotype.

**Meiotic arrest and impaired meiotic recombination in Ing2−/− testes**

DNA contents were analyzed in cells from 2- and 6-month-old Ing2+/+ and Ing2−/− testes (Fig. 2G). Ing2−/− testes showed reduced 1N fractions (representing round and elongated spermatids) at 2 months of age, which were almost absent at 6 months. A relative increase in 2N fractions (spermatogonia and somatic cells) in Ing2−/− testes can be attributed to a failure to complete meiosis II as well as to the presence of somatic cells associated with Leydig cell hyperplasia and Sertoli-cell-only tubules in degenerated seminiferous tubules. An apparent loss of 4N fractions (spermatocytes) in Ing2−/− testes became evident at 6 months that can be
Figure 1. Testicular atrophy and semen abnormalities in Ing2−/− mice. (A) ING2 is abundant in testes. qRT-PCR analysis of ING2 expression in mouse tissues. ING2 expression levels (normalized to GAPDH) are shown on a scale of 10−4. (B) Immunohistochemical (IHC) staining of ING2 protein in a normal human testis section. Positive nuclear staining is evident in the seminiferous tubule. Scale bar is 100 μm. (C) Morphology of reproductive organs from 8-week-old Ing2+/+ and Ing2−/− male mice. The seminal vesicles (black arrowheads), bladder (white arrows), epididymis (white arrowheads), testes (black arrows) are demonstrated. Scale bar, 1 cm. (D) Testis weight during postnatal development of Ing2+/+ and Ing2−/− mice. (E) Normal sperm count of Ing2+/+ and Ing2−/− mice. (F) Normal sperm count of Ing2+/+ and Ing2−/− mice.
attributed to premature loss of germ cells with ageing. Immuno-histochemical (IHC) staining of phosphorylated histone H2AX (γ-H2AX) marked DNA double-strand breaks at leptotene stage associated with meiotic recombination [21,22] in both Ing2+/+ and Ing2−/− testes [Fig. 2H-K, black arrows in (I) and (K)]. The γ-H2AX signals were diminished at pachytene stage in Ing2+/+ seminiferous tubules [Fig. 2H,I; white arrows in (I)], as normally expected with the completion of meiotic recombination [21,22]. In contrast, Ing2−/− seminiferous tubules were defective in progression to the γ-H2AX-negative pachytene stage [Fig. 2J,K] and instead contained spermatocytes with abnormally accumulated γ-H2AX [Fig. 2K, white arrowheads]. These results are consistent with the histopathological observations described above, and indicate a failure to complete meiosis, as well as a progressive loss of germ cells with ageing in Ing2−/− mice.

Gene expression profiles in Ing2−/− testes are consistent with spermatogenesis arrest

Gene expression profiling by mRNA microarray analysis using whole testis RNA identified 619 genes differentially expressed (381 downregulated and 238 upregulated, P<0.001, FDR<0.04) in Ing2−/− testes compared with Ing2+/+ testes (GSE18610 at http://www.ncbi.nlm.nih.gov/geo/). Ing2 was the most downregulated gene (Table S1), confirming the validity of the experimental system. A gene list consisting of genes differentially expressed in Ing2−/− testes was used to query a database of gene expression profiles during mouse spermatogenesis, including spermatogonia, spermatocytes and spermatids, as well as Sertoli cells, whole seminiferous tubules, and whole testes [GermOnline, http://www.germonline.org [23]]. There was a significant correlation (non-parametric Spearman correlation P<0.0001) between gene expression in Ing2−/− testes and gene expression during mouse spermatogenesis. Specifically, genes differentially expressed in Ing2−/− testes were positively correlated with genes expressed in Sertoli cells (Spearman r = 0.72 and spermatogonia (Spearman r = 0.70), but negatively correlated with genes expressed in spermatocytes (Spearman r = −0.25) and spermatids (Spearman r = −0.74). These expression profiles further validate the conclusions from our histopathological and IHC findings that indicate failed differentiation of spermatocytes as the major defect caused by Ing2 deficiency.

| Table 1. Weight of organs, serum testosterone levels, and sperm parameters in Ing2+/+ and Ing2−/− male mice. |
|----------------------------------|------------------|------------------|
| **Ing2+/+**                      | **Ing2−/−**      | **P value**      |
|----------------------------------|------------------|------------------|
| Total body weight (g)b           | 25.9±4.0         | 23.6±1.4         | NS               |
| Organ Weight (mg)c               | 321±10.8         | 312±8.6          | NS               |
| Brain                            | 79.2±30.1        | 59.6±6.8         | NS               |
| Thymus                           | 86.1±6.3         | 76.1±14.6        | NS               |
| Lung                             | 153±16.9         | 143±37.7         | NS               |
| Heart                            | 103±19.8         | 79.4±7.7         | NS               |
| Spleen                           | 192±27.3         | 167±12.9         | NS               |
| Kidney                           | 1539±432         | 1353±176         | NS               |
| Liver                            | 234±32.3         | 221±77.4         | NS               |
| Stomach                          | 203±3±4848       | 2027±448         | NS               |
| Seminal vesicle                  | 105±19.4         | 83.1±11.1        | NS               |
| Bladder and prostate             | 132±22.7         | 108±19.0         | NS               |
| Epididymis                       | 19.5±2.6         | 17.9±7.1         | NS               |
| Testis                           | 91.8±13.6        | 45.2±11.2        | 0.0004           |
| Serum testosterone (ng/dl)d      | 8 wks            | 88.6±22          | 135±48           | NS               |
|                                  | 26 wks           | 66.4±17          | 44.8±19          | NS               |
| Sperm parameters**               | Total no. of cells(x10⁶) | 44.4±5.8         | 9.8±1.7          | 0.005            |
|                                  | No. of normal sperm (x10⁶) | 32±7.5           | 0.7±0.2          | <0.0001          |
|                                  | Sperm motility (%) | 91.0±4.3         | 11.4±3.2         | <0.0001          |

aStudent’s t test, NS; Not significant.
b8-week-old mice were examined, n = 5 per group. Values are means ± SD.
cThe wet weights of paired organs were averaged for each mouse, and this single value was used to calculate mean ± SD among same genotype.
dn = 5 per group. Values are means ± SD.
eStudent’s t test, n = 5 per group. Error bars are s.e.m.
fGermOnline, http://www.germonline.org [23].

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Figure 2. Degeneration of seminiferous tubules and meiotic arrest in Ing2<sup>−/−</sup> testes. (A–E) Histological analysis of testis sections from 8-week-old Ing2<sup>+/+</sup> (A, B) and Ing2<sup>−/−</sup> (C–E) mice by hematoxylin and eosin (H&E) staining. Black arrows in (B) and (D) indicate spermatocytes with large, condensed nuclei. The white arrow in (B) indicates spermatids, which are missing in (D). In (E), Leydig cell hyperplasia (white arrows), apoptotic cell (black arrow) and multinucleated giant cell (white arrowhead) are indicated. Scale bars are 200 μm in (A) and (C), 100 μm in (B), (D) and (E, left), and 50 μm in (E, right). (F) Histological analysis of epididymis from 8-week-old Ing2<sup>+/+</sup> and Ing2<sup>−/−</sup> mice by H&E staining. Scale bars, 100 μm. Black arrows indicate degenerated round cells in Ing2<sup>−/−</sup> epididymis. (G) Flow cytometric analysis of testis cells isolated from 2- and 6-month-old Ing2<sup>+/+</sup> and
ING2 Fosters Spermatogenesis and Tumor Suppression

Downregulation of genes encoding spermatogenesis-related proteins and chromatin regulatory factors in Ing2+/−/− testes

A subset of differentially expressed genes identified by microarray analysis that have well-characterized roles in spermatogenesis and chromatin regulation is listed in Table S1. A group of genes involved in normal differentiation and function of spermatids or sperms, including Prox2 [24], Slh [25], Step2 [26], Sry [27] and the Spo11 family of testis-specific genes [28], were downregulated in Ing2−/−/− testes, consistent with impaired progression to these later stages of male germ cell development. Two downregulated genes, Abhd4 (ankyrin repeat and SOCS box-containing protein 4) [29] and Gzmnb (granzyme N) [30], are believed to be specifically involved in spermatocyte differentiation at pachytene stage, when Ing2 deficiency manifested its effect. The marked downregulation of these two genes in Ing2−/−/− testes was validated by qRT-PCR (Fig. S4). It should also be noted that several genes encoding chromatin modifying or associated proteins, including Setd2 [31], Zfp57 [32], Suz39h2 [12], Sath2 [33], Ing3 [34] and Phf21a [35], were downregulated in Ing2−/−/− testes. In addition to the deregulation of chromatin modifications by loss of Ing2 itself, decreased expression of these proteins may possibly affect chromatin status and contribute to defective spermatogenesis in Ing2−/−/− testes. A set of somatic cell-derived transcripts upregulated in testes of HDAC inhibitor-treated mice [14] were not significantly changed in Ing2−/−/− testes (Table S2).

Aberrant chromatin modifications in Ing2−/−/− testes

The amounts of H3K4me3, HDAC1 and mSin3A, which all functionally interact with ING2 to regulate chromatin modification [6,7], were found to be abundant in the testes in Ing2+/++/+ mice (Fig. S5). In IHC staining, both Ing2+/+ and Ing2+/−/− testes contained HDAC1-negative spermatogonia and leptotene spermatocytes (Fig. 3A, leftmost panel; black arrows). HDAC1 was induced during spermatocyte differentiation into pachytene stage in Ing2−/−/− testes (Fig. 3A, leftmost panel; white arrow). In contrast, spermatocytes in Ing2−/−/− testes showed no significant HDAC1 staining (Fig. 3A, leftmost panel; white arrowheads), consistent with the impaired differentiation to the pachytene stage. Total amounts of HDAC1 were also shown to be decreased in Ing2−/−/− testes by Western blot (Fig. 3B,C). Three acetylation sites on core histones (H3K18, H4K8 and H4K12) were acetylated in spermatogonia and leptotene spermatocytes in both Ing2+/+ and Ing2−/−/− testes (Fig. 3A, right three panels each; black arrows). In Ing2−/−/− testes, the deacetylation of these lysine residues was coincident with HDAC1 induction (Fig. 3A, right three panels; white arrowheads). In contrast, lack of HDAC1 induction in Ing2−/−/− seminiferous tubules resulted in accumulated spermatocytes with these sites highly acetylated (Fig. 3A, right three panels; white arrowheads). These results suggest that the meiotic arrest before pachytene stage due to Ing2 deficiency is associated with impaired accumulation of HDAC1 and deregulated histone acetylation.

p53-dependent and independent apoptosis in Ing2−/−/− testes

Because of the functional association of Ing2 with p53 and the well-characterized role of p53 in regulating apoptosis in the testis [36,37,38], we next explored the effect of Ing2 deficiency on p53 expression. To this end, 8-week-old Ing2−/− and Ing2−/−/− testes, as well as p53−/−/− testes as a negative control, were examined for p53 protein expression. A ~2.5-fold increase in p53 protein was observed in whole testis lysates of Ing2−/−/− mice compared to age-matched Ing2+/+ mice (Fig. 4A,B). Immunohistochemical analysis of p53 protein revealed intratubular staining only in germ cells of Ing2−/−/− testes, but not Ing2+/+ or p53−/−/− testes (Fig. 4C). No significant increase in p53 mRNA level was found in Ing2−/−/− testes (Fig. 4D) suggestive of a post-transcriptional mechanism for p53 induction. PUMA, a p53-induced apoptosis effector, was also upregulated in luminal regions of the tubules in Ing2−/−/− testes (Fig. S6). These results indicate that endogenous ING2 promotes germ cell survival and differentiation. Loss of Ing2 leads to p53 activation in vivo, perhaps as an indirect result of testicular degeneration or through a novel regulatory interaction between p53 and ING2.

To better characterize the tubular degeneration brought about by Ing2 deficiency, we determined the presence of apoptotic or senescent germ cells in testes at 8 weeks of age. We found that Ing2−/−/− testes had significantly increased numbers of TUNEL-positive tubules and TUNEL-positive cells per tubule, compared with those in age-matched Ing2+/+ testes (Fig. 5A, left two bars in both panels). While apoptotic germ cells were observed rarely and close to the basement membrane in Ing2−/−/− testes (Fig. 5B, Ing2+/+), they occurred frequently in spermatocytes in luminal regions of the tubules (Fig. 5B, Ing2−/−/−). Neither Ing2+/+ nor Ing2−/−/− testes at 8 week of age showed positive staining for senescence-associated β-galactosidase, a marker of cellular senescence [39], in their seminiferous tubules (data not shown).

We next investigated whether disruption of p53 would rescue defective spermatogenesis and enhanced apoptosis of Ing2−/−/− mice. Although spermatocytes in p53−/−/− males may have an impaired DNA damage response, leading to the formation of a multinucleated giant cell [37,40], they show otherwise normal spermatogenesis and are fertile [41] (Fig. 5C, p53−/−/−), allowing us to generate p53/+ Ing2−/− double-knockout mice. Ing2 deficiency in a p53−/−/+ background reproduced the abnormalities observed in the presence of p53, including reduced testis weight (Fig. 5D), degeneration of seminiferous tubules, hyposperma in epididymides, reduced numbers of normal spermatooza in semen (Fig. 5C, p53−/−/− Ing2−/−) and enhanced apoptosis (Fig. 5A, right two bars in both panels; Fig. 5B, p53−/−/− and p53−/−/− Ing2−/−). However, these abnormalities in p53−/−/− Ing2−/− males were less severe than those in Ing2−/−/− males as mentioned above. Quantitative data analysis of the TUNEL assay showed that p53−/−/− Ing2−/− testes had higher levels of apoptosis than Ing2−/−/− and p53−/−/− testes but lower levels of apoptosis than Ing2−/−/− testes (Fig. 5A), suggesting that Ing2 deficiency induces apoptosis in a p53-dependent manner, which is consistent with p53 induction in Ing2−/−/− testes (Fig. 4), as well as in a p53-
independent manner. Thus, p53 deficiency partially rescued the pathological changes due to Ing2 deficiency. Nevertheless, degenerated large, round cells were accumulated in p53<sup>−/−</sup>Ing2<sup>−/−</sup> epididymis and semen (Fig. 5C; black arrows), and p53<sup>−/−</sup>Ing2<sup>−/−</sup> males were still infertile.

Reduced ING2 expression is associated with impaired spermatogenesis and male infertility in humans

To examine the expression of ING2 in men with low sperm count, morphologically abnormal sperm and/or impaired spermatogenesis, public microarray datasets available at GEO (http://www.ncbi.nlm.nih.gov/geo) and ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae) were queried using the Nexbio search engine (http://www.nexbio.com) (Table 2). Two datasets (GSE6967 and GSE6872) comparing spermatooza purified from semen samples of infertile teratozoospermic men versus normal fertile men [42] showed significantly lower levels of ING2 expression in the former cases. In two independent studies comparing testicular biopsies with impaired spermatogenesis classified according to Johnsen score [43] versus full spermatogenesis (GSE4797 and five comparisons in E-TABM-234) [44,45], decreased expression of ING2 was consistently associated with the spermatogenic defect. Furthermore, ING2 expression was lower in testicular biopsy specimens from non-obstructive azoospermia (NOA) patients than those from obstructive azoospermia (OA) patients (GSE9210) [46], suggesting the functional involvement of reduced expression of ING2 in human male infertility due to defective germ cell development, but not that due to a physical obstruction. Considering that germ cells are the major source of ING2 expression in tests, the finding of low ING2 expression in men with Sertoli-cell only syndrome likely reflects the fact that the spermatogenic cell types are absent from the seminiferous tubules. This association does not per se indicate a causative role for ING2 deficiency in human male infertility.
Figure 4. Induction of p53 in Ing22−/− testes. (A–B) Western blot analysis of p53 protein expression using whole testis lysates from Ing22+/+ and Ing22−/− testes, as well as p532−/− testes (negative control), at 8 weeks of age. β-actin was a loading control. Relative p53 expression levels (normalized to β-actin) based on quantitative image analysis are shown in (A) and in (B). Student’s t test. n = 3 per group. Error bars are s.e.m. (C) p53 staining in testes from 8-week-old p532−/−, Ing22+/+ and Ing22−/− mice. Black arrows indicate intratubular positive staining in Ing22−/− testes. Both Ing22+/+ and Ing22−/− testes, as well as p532−/− testes to a much lesser extent, show interstitial staining (white arrowheads). Scale bars, 100 µm. (D) Real-time qRT-PCR analysis of p53 mRNA expression. Two independent primers/probe sets (assays 1 and 2) were used. The expression levels in Ing2−/− testes are shown as the relative values to those in Ing2+/+ testes. Data are mean ± s.e.m. from n = 8 (Ing2+/+) or n = 14 (Ing2−/−). No statistically significant difference was observed (Student’s t test).

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Figure 5. Increased apoptosis and defective spermatogenesis in testes by Ing2 deficiency in the absence of p53. (A, B) TUNEL assay in testes from 8-week-old Ing2+/+, Ing2−/−, p53−/− and p53−/− Ing2−/− mice. (A) The data are shown as ratios of TUNEL-positive tubules to total tubules examined (left) and as average numbers of TUNEL-positive cells per a TUNEL-positive tubule (right). At least 50 tubules were examined in each group.
**ING2 Fosters Spermatogenesis and Tumor Suppression**

**Ing2 is a tumor suppressor gene**

To examine whether Ing2 deficiency affects tumorigenesis and the aging processes, we observed Ing2+/+ (n = 22) and Ing2−/− (n = 28) mice for two years. There was no statistically significant difference in survival between the two genotypes (Fig. S7, P = 0.43, Log-rank test). However, detailed histopathological analysis of both groups at time of death showed a significant change in malignant tumor spectrum. The incidence of soft tissue sarcomas was increased in Ing2−/− mice (P = 0.017) (Table 3). The major tumor type observed in Ing2−/− mice was histiocytic sarcoma (Fig. S8, Table 3), which showed increased incidence preferentially in males for currently unknown reasons (Table S3). Although we confirmed significantly increased frequencies of degenerated seminiferous tubules and oligozoospermic epididymis in Ing2−/− mice (Tables S4 and S5), pathological examinations of other organs and tissues showed that Ing2−/− mice had no remarkable difference from Ing2+/+ mice in the spectrum of non-malignant lesions, except for increases in benign hardnerian gland adenomas and in atypical lymphoid hyperplasia of the spleen and a decrease in acinar dilation in prostate (Table S4). Ing2−/− mice also showed no signs of premature aging, such as hair graying, alopecia, skin atrophy, lordokyphosis, osteoporosis or cataracts. Thus, we currently conclude that Ing2 deficiency by itself does not affect aging phenotypes but enhances spontaneous formation of soft-tissue sarcomas.

**Discussion**

This study provides evidence that ING2 is an essential regulator of mammalian spermatogenesis by showing that: 1) testes express high levels of ING2 (Fig. 1A,B, Fig. S1); 2) genetic knockout of Ing2 causes a spermatogenesis defect and male infertility in mice (Figs 1C–G, 2A–F and Table 1); and 3) decreased ING2 expression is highly associated with defective spermatogenesis and male infertility in humans (Table 2). The data indicate that the spermatogenic function of ING2 depends on both its regulatory effect on chromatin (Fig. 3) as well as its functional interaction with p53 (Fig. 4, 5).

The spermatocytes in defective Ing2−/− seminiferous tubules underwent meiotic arrest (Fig. 2G), which was also observed in mouse models deficient for histone methyltransferases [10,11,12]. However, unlike these mouse models in which fertility was impaired in both male and female, Ing2 deficiency only affected male fertility, suggesting a distinct role for ING2 in regulating chromatin modification during mammalian germ cell development. Histopathologically, the blocked differentiation of Ing2−/− spermatocytes into pachytene stage (Fig. 2H–K,5A) most resembled the meiotic defect in mice treated with a HDAC1 inhibitor, trichostatin-A [15]. The ability of ING2 to recruit HDAC1 to H3K4me3 [6], the dynamic regulation of H3K4me3 during zygotene-to-pachytene progression [17], and the accumulation of HDAC1 and the deacetylation of core histones in pachytene spermatocytes in Ing2−/− but not Ing2−/− mouse testes (Fig. 3A) all suggest that the spermatogenesis defect by Ing2 deficiency is due to a disturbance of the stage-specific histone modifications coordinated by the H3K4me3-ING2-HDAC1 interaction. Our data establish ING2 as a chromatin-associated, non-enzyme protein that is critical to temporal and spatial profiles of chromatin modifications during spermatogenesis. A recent study provides further support to this hypothesis. Suberoylanilide hydroxamic acid (SAHA), an HDAC inhibitor used clinically for the treatment of cancer causes the dissociation of ING2 from the Sin3 deacetylase complex leading to de-repression of downstream genes and growth inhibition[47]. Our findings reveal a plausible mechanism by which HDAC inhibitors may disrupt deacetylase function during spermatogenesis, through disruption of Ing2-bound co-repressor complexes. Consistent with this is also the finding that mice deficient on the Sirt1 protein deacetylase show spermatogenesis defects similar to Ing2-deficient mice [48]. SIRT1 is recruited by ING proteins to negatively regulate mSin3A/

**Table 2. ING2 expression in spermatogenic pathologies in humans.**

| Dataset ID (ref) | Specimen | Control group | Test group | Fold change | P value |
|-----------------|----------|---------------|------------|-------------|---------|
| GSE6967 (42)    | Sperm    | Normal        | Teratozoosperm | −67.4       | 0.0008  |
| GSE6872 (42)    | Sperm    | Normal        | Teratozoosperm | −3.20       | 2.4e-5  |
| GSE4797 (44)    | Testicular biopsies | Full spermatogenesis | Sertoli-cell-only syndrome | −7.75       | 0.0112  |
| E-TABM-234 (45) | Testicular biopsies | Full spermatogenesis | Johnsen score 2d | −1.52       | 0.0025  |
|                 |          |               | Johnsen score 3.2d | −1.70       | 0.0006  |
|                 |          |               | Johnsen score 5d  | −1.62       | 0.0012  |
|                 |          |               | Johnsen score 7d  | −1.45       | 0.0052  |
|                 |          |               | Johnsen score 8d  | −1.32       | 0.0267  |
| GSE9210 (46)    | Testicular biopsies | Obstructive Azooospermia | Non-obstructive Azooospermia | −1.34       | 0.0009  |

*Log2 ratio (Test group/Control group).  
**Welch’s t test.  
†Equivalent to Johnsen score 2.  
‡Johnsen score 2: no germ cells, Sertoli cells only; Johnsen score 3.2: Sertoli cells and few spermatogenesis; Johnsen score 5: no spermatids, many spermatocytes; Johnsen score 7: no late spermatids, many early spermatids; Johnsen score 8: few late spermatids.

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**Table 3.** Malignant tumors arising in aging study.

| Tumor Type                        | Ing2+/− (n=22) | Ing2−/− (n=28) | P value
|-----------------------------------|----------------|----------------|---------|
| Soft-tissue sarcomas              |                |                |         |
| Histocytic sarcoma                | 3/22 (14%)     | 13/28 (46%)    | 0.017   |
| Hemangiosarcoma                   | 1              | 8              |         |
| Leiomyosarcoma                    | 1              | 2              |         |
| Sarcoma, NOS                          | 0              | 2              |         |
| Neurofibrosarcoma                  | 0              | 1              |         |
| Lymphomas                         | 4/22 (18%)     | 2/28 (7%)      | NS      |
| Follicular B-cell lymphoma         | 2              | 2              |         |
| Splenic marginal zone B-cell lymphoma | 2              | 0              |         |
| Carcinomas                        |                |                |         |
| Lung                              | 7/22 (32%)     | 8/28 (29%)     | NS      |
| Duodenum                          | 6              | 3              |         |
| Ovary                             | 1              | 0              |         |
| Liver                             | 0              | 1              |         |
| Pancreas                          | 0              | 1              |         |
| Thyroid                           | 0              | 1              |         |
| Osteosarcomas                     | 0/22 (0%)      | 0/28 (0%)      | NS      |

*10 female and 12 male.
*11 female and 17 male.
*1 Fisher’s exact test; NS: Not significant.
*2Not otherwise specified.

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HDAC1 transcriptional repression activity [49]. It is unknown whether an altered expression of one or a few specific genes or a genome-wide change in chromatin status mediates the defects in Ing2-deficient mice, as well as in other infertile mice deficient for chromatin modifying factors. The two pachytene-specific genes identified in this study, Asb4 and Gzmz (Fig. S4), deserve further investigation. The somatic cell-derived transcripts differentially expressed in testes of trichostatin-A-treated mice [14] were not significantly affected by Ing2 deficiency (Table S2), in agreement with the findings that germ cells are the major cell type expressing Ing2 [Fig. 1B, Fig. S1]. Ing2-deficient mice should thus be a suitable model to study germ cell-autonomous effects of chromatin modifications on spermatogenesis.

Ing2−/− mice exhibited phenotypic differences from mice deficient for Ing1, the founder member of the ING family, which could be explained by the difference in organ-specific expression profiles [20], the different interacting proteins [1], the different modes of functional interaction with p53 [50], and/or the different p33-independent functions [51]. Ing1 expression was low in testes [20] and Ing1-deficient mice were fertile [51,52]. Whereas Ing1−/− mice had reduced body size [52], Ing2−/− mice grew normally. Furthermore, while Ing1−/− mice had elevated incidence of B-cell lymphomas [51,52], Ing2−/− mice had elevated incidence of histiocytic sarcoma (Table 3, S3). p19Arf-deficient mice exhibit similar phenotypes to Ing2−/− mice, such as testicular atrophy, increased germ cell apoptosis, loss of sperm and high incidence of histiocytic sarcoma [53,54]. This most intriguing finding brings up the possibility that Ing2 and p19Arf, two p33 regulatory proteins, are involved in a common pathway that is aimed to restrict self-renewal and ensure normal differentiation during spermatogenesis as well as prevent tumor development.

p53 regulates apoptosis in testes, which eliminates germ cells with DNA damage induced by irradiation [36,37] or spontaneously occurring during normal spermatogenesis [38]. p33-independent apoptosis also occurs during spermatogenesis in response to DNA damage and meiotic arrest [37,55]. Our comparative analysis of apoptosis in Ing2+/−, Ing2−/−, p33−/−, and p33−/−/Ing2−/− tests (Fig. 5A,B) indicates that both p33-dependent and independent mechanisms of apoptosis were activated by Ing2 deficiency, probably contributing to the elimination of developmentally arrested spermatocytes with unprocessed DNA lesions (Fig. 2J,K). The induction of p53 by Ing2 deficiency (Fig. 4) suggests that a physiological in vivo function of endogenous ING2 may be to prevent an illegitimate activation of p33 under non-stressed conditions. However, this functional link between Ing2 and p33 is not a sole mechanism by which ING2 ensures normal spermatogenesis and male fertility, as indicated by the mitigated but still significant pathological changes in testes (Fig. 5C,D), the accumulation of abnormal spermatozoa (Fig. 5C) and infertility in p33−/−/Ing2−/− males.

Approximately 10% of couples suffer from infertility with approximately equal contributions from men and women [56]. Meiotic arrest during spermatogenesis, as observed in Ing2−/− mice, is frequently associated with male infertility in humans [57]. However, because of the complexity of the spermatogenic process possibly involving thousands of different genes, a majority of male infertility cases in humans remain not understood [19]. Given a disturbed pattern of chromatin modification in defective spermatogenesis in humans [18] and decreased Ing2 expression commonly associated with sperm abnormalities and pathological changes in spermatogenesis in humans (Table 2), we propose that the Ing2-mediated chromatin regulation is critical to normal meiotic progression during spermatogenesis in humans as well and may be widely, if not universally, impaired by a number of different spermatogenic defects leading to idiopathic infertility in men. Genetic and epigenetic changes, as well as functional
variants, of the ING2 gene itself also deserve investigation in infertile men.

This study also has significant implications in infertility in men with other diseases, in particular malignant tumors. Patients with testicular cancers are frequently infertile [58,59], with the degeneration of peritumoral, non-cancerous seminiferous tubules [10,30]. HDAC inhibitors are under clinical trial in patients with various types of solid and hematologic malignancies [90,61]. Spermatogenesis is a process highly sensitive to DNA damage by common chemotherapy and radiotherapy [38,62] and male infertility is a major quality-of-life issue in cancer survivors [58,63]. We expect that the Ing2<br><br>-/- mice generated and characterized in this study can be a model system to study idiopathic and iatrogenic male infertility in humans.

**Materials and Methods**

**Generation of Ing2<sup>-/-</sup> mice**

This mice study was approved by the ACUC, NCI-Frederick and all the guidelines were followed for the study. To construct the targeting vector for Ing2, a BAC clone containing the Ing2 locus was isolated from a 129-mouse genomic library (Genome Systems Inc., St.Louis, MO). A 6.2-kilobase (kb) HindIII–HindIII fragment encompassing from the first intron to the second intron was cloned into the HindIII site of the pBS-SK<sup>+</sup>I site of the pLoxpneo vector (a gift from Dr. Chuxia Deng) [64]. A single loxp site was inserted into the pBSI site at the first intron within the 6.2-kb fragment cloned in the pBS-SK<sup>-</sup>I and XhoI site inserted into the XhoI and XhoI sites of the pLoxpneo containing the 1.7-kb fragment. The final targeting construct was designated pLoxpneoING2 (Fig. S1a). The targeting construct was linearized and electroporated into ES cells derived from 129/Sv mice. G418-resistant colonies were selected and expanded. The ES clones with correct targeting events were identified by Southern blot and PCR. Heterozygous mice with the targeted allele (Ing2<sup>lox<sub>few-<sup>xox</sub>/><sub>few</sub>burst</sup>Ing2<sup>z<i>xox</i></sub>) were crossed with each other, resulting in homozygous mice (Ing2<sup>z<i>xox</i></sub> bur<sub>z<i>xox</i></sub>ing2<sup>z<i>xox</i></sub>) resembling mice were crossed with EIIa-Cre[65] mice to remove DNA fragments between two loxp sites. This led to the generation of three types of offspring with a deletion of the neomycin resistance cassette (Ing2<sup>lox</sup>tho), the exon 2 (Ing2<sup>z<i>xox</i></sub>), or both (Ing2<sup>-/-</sup>). The identified Ing2<sup>-/-</sup> mice were then bred with C57BL/6J mice to segregate the EIIa-Cre transgene. Ing2<sup>-/-</sup> mice without EIIa-Cre were mated with each other, leading to the generation of Ing2<sup>-</sup> knockout mice (Ing2<sup>-/-</sup>). To genotype mice, genomic DNAs were extracted from tails using REDExtract-N-Amp Tissue PCR kit (Sigma, St. Louis, MO) and analyzed by PCR. Thermal cycling was carried out for 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. The primers were as follows: Ing2-F1 (5′-agtcttcagagcacatta-3′) commonly used for ing2<sup>-</sup> and ing2<sup>-</sup>-loci, Ing2-R1 (5′-gtgctcctgtaggcag-3′) for amplifying 118-bp product from ing2<sup>-</sup> locus, and Ing2-R3 (5′-gattcctgtaggcag-3′) for amplifying 156-bp product from Ing2<sup>-</sup> locus. p53-deficient mice were previously described [41], p53<sup>-/-</sup> and Ing2<sup>-/-</sup> double knockout mice (p53<sup>-/-</sup>/ing2<sup>-/-</sup>) were generated as previously described by Hussain et al [66] for p53<sup>-/-</sup> and NOS2 double knockout mice.

**Quantitative real-time RT-PCR**

Total RNAs were extracted from the whole testes of 8-week-old mice using Trizol (Invitrogen) according to the manufacturer’s protocol. Five micrograms of total RNA were used for the synthesis of first strand cDNA using the SuperScript III First Strand cDNA Synthesis Kit (Invitrogen). To examine Ing2 expression in various mouse organs, BD MTC Mouse Panel III was purchased from BD Biosciences (San Jose, CA). Real-time RT-PCR analysis was performed using ABI prism 7900 (Applied Biosystems, Foster City, CA) with Taqman Gene Expression Assays, purchased from Applied Biosystems; Ing2 (Mm00469833_m1), p53 (Mm00441964_g1, assay 1 in this study), p53 (Mm01337166-mH, assay 2 in this study), Asb4 (Mm0040830_m1), and GzmB (Mm00461580_m1). GAPDH (Applied Biosystems, Mm99999915_g1) was used as internal control. Normalized or relative gene expression was calculated using the equation 2<sup>-ΔΔCt</sup> or 2<sup>-ΔCt</sup>, respectively, where ΔCt = Ct(Gene)–Ct(GAPDH).

**Gross anatomical and histological examinations**

Gross examination of organ weight and morphology on all mice necropsied at 2 weeks to 2 years of age. For routine histopathological examination all tissues were formalin-fixed, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin (H&E).

**Western blotting**

Testes and other organs were removed from 8-week-old mice. Mouse embryo fibroblasts were prepared by standard procedures. For Western blotting, tissues and cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 1 mM EDTA, 0.1% SDS, 10 mM Tris-HCl, pH 7.5) containing complete protease inhibitors (Roche, Indianapolis, IN). Protein samples were run on SDS-polyacrylamide gels (4–20% gradient) (Invitrogen) and blotted onto 0.45-μm nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked with 5% skim milk in Tris-buffered saline before incubation with primary antibodies: anti-HDAC1 (Millipore, Billerica, MA), anti-H3K4me3 (Abcam, Cambridge, MA), anti-mSin3A (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p53 (Novus Biologicals Inc), anti-p53 (Santa Cruz Biotechnology, and anti-ING2 [68]. Signals were detected according to standard procedures using ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ). Quantitative image analysis of relative protein expression levels was performed using ImageJ 1.40 g software (http://rsb.info.nih.gov/ij/).

**Immunohistochemical Staining (IHC)**

For IHC, tissues were fixed in formalin. Antigen retrieval and immunohistochemical staining were performed as previously described [67] using EnVision System-HRP (DAB) (Dako Cytomation, Carpinteria, CA). Antibodies used for IHC were as follows: anti-AcH3K18 (Cell Signaling Technology, Danvers, MA), anti-AcH4K8 (Cell Signaling Technology), anti-AcH4K12 (Cell Signaling Technology), anti-γ-H2AX (Novus Biologicals Inc, Littleton, CO), anti-HDAC1 (Millipore), anti-p53 (Novocasta, Bannockburn, IL) and anti-ING2 (Sigma).

**TUNEL assay**

For terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) analysis, formalin-fixed sections were deparaffinized, rehydrated, and pretreated with proteinase K. Apoptotic cells were detected using DeadEnd Colorimetric TUNEL system kit (Promega, Madison, WI) according to the manufacturer’s instructions.
Sperm counts and motility

For each mouse (8-, 16- and 24-week-old) whole epididymis and vas deferens were harvested, cut into 2-mm-long pieces, resuspended in 1 ml of buffer containing 75 mM NaCl, 24 mM EDTA and 0.4% bovine serum albumin. Pieces and sperm fluid were homogenized at 32°C for 10 min to dissociate somatic cells. Sperm cells remaining as a monodispersed suspension were counted on a hemocytometer.

Testosterone assay

Blood was taken from 8- and 26-week-old male mice (five in each group) that were being housed singly, in separate cages. Female mice were being housed in separate cages of the same room at the time of blood sampling. Serum testosterone level was measured using ELISA kit (Alpha Diagnostic International, San Antonio, TX) according to the manufacturer’s instructions.

Flow cytometry

Testes were excised from 2- and 6-month-old mice (three mice in each group), decapsulated and crushed through 20-gauge needles and 70-μm cell strainers (BD Biosciences) in phosphate-buffered saline. Cells (2×10⁶) were treated with RNase and stained with propidium iodide using a Cycle Test Plus DNA reagent kit (Becton Dickinson, Franklin Lakes, NJ). All fluorescence-activated cell sorting data were analyzed using CELL Quest (version 3.3; Becton Dickinson).

mRNA microarray analysis

Total RNA samples were isolated from whole testes of Ing2⁺/⁺ (n = 3) and Ing2⁻/⁻ (n = 5) mice at 2-3 months of age using Trizol (Invitrogen). Microarray analysis was performed using Affymetrix platform (Mouse Gene 1.0 ST Array). Data was RNA-normalized using Affymetrix Expression Console, annotated according to NetAffx release: 28 (2009-03-16) and summarized at the gene level. Resulting normalized and annotated probesets were imported into BRB Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.html). A Class Comparison analysis, based on a Two-sample T-test using the random variance model, yielded 1046 significantly differentially expressed genes (FDR <0.05, P <0.001). Those that were not associated with a gene name were excluded from further analysis. After further filtering for multiple occurrences of a gene ID, 619 unique genes remained, of which 381 were upregulated and 238 were downregulated.

Bioinformatics analyses

To determine a cell type-specific expression of ING2 in mouse testis, Ing2 mRNA expression values were extracted from the public Gene Expression Omnibus data repository (GEO, http://www.ncbi.nlm.nih.gov/geo/). A microarray dataset of testes from C57BL/6 mice (accession number GDS409) was interrogated. The expression values for ING2 probesets were identified within the normalized data files available at GEO, and they were subsequently plotted to generate graphical displays.

To examine whether ING2 expression levels are associated with spermatogenic defects and male infertility in humans, we used the datasets available at GEO and ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/), which encompass microarray analysis of testes from men with low sperm count, morphologically abnormal sperm and/or impaired spermatogenesis. Meta-analysis was performed using the Nextbio database of curated and processed high-throughput data (http://www.nextbio.com) by querying for the search term “ING2” and filtering for “male infertility” or “azoospermia”. The analysis output consisted of individual microarray study results that showed differential ING2 expression for the tested comparisons in each dataset. For each microarray study, the fold change and p-value of ING2 for the comparison were indicated.

To compare between the existing datasets of spermatogenesis-associated profiles of gene expression and the differentially expressed genes identified by the mRNA microarray analysis in this study, the gene symbols were compiled into two genesets: upregulated (238) or downregulated (381) in Ing2⁻/⁻ mice. These genesets were separately uploaded to GermOnline (http://www.germonline.org [23]), a database of microarray expression profiling data from experiments relevant for the mitotic and meiotic cell cycle, gametogenesis and fertility, from which corresponding expression values were extracted. Expression intensities of 90 and 138 genes were extracted from the upregulated and downregulated genesets, respectively. Expression intensities in Sertoli cells, spermatogonia, spermatocytes, spermatids and tubules were normalized to total testis and plotted separately for each geneset.

Analysis of spontaneous tumors

Life span and spontaneous tumor incidence were determined in Ing2⁺/⁺ and Ing2⁻/⁻ mice. Mice with visible tumors and moribund mice showing weight loss or difficulties in moving were sacrificed upon detection. The remaining surviving mice were sacrificed at 24 months of age. Gross anatomical and histological examinations were performed as described above.

Human subjects

The histology of Mouse testis was compared with human testis obtained from the Armed Forces Institute of Pathology at Water Reed Army Medical Center (AFIP). Use of the human organs was approved by the NIH Office of Human Subjects Research (OHSR) which issued an IRB Exemption (#4545) for the study. Immunohistochemical (IHC) staining of ING2 protein in a normal human testis section was done at the AFIP at Water Reed Army Medical Center.

Supporting Information

Figure S1 IHC staining of ING2 in normal human testis. The rectangular area in (A) is enlarged in the (B). Scale bars are 200 μm in (A) and 100 μm in (B). The specimens in this figure and in Fig.1B were obtained from different individuals. (TIF)

Figure S2 Generation of Ing2⁻/⁻ mice. (A) Schematic representation of the targeting vector [pLoxPneoIng2], the wild-type Ing2 locus and the targeted locus. Ing2 exon 1, Ing2 exon 2 and neomycin-resistant gene cassette (TA/neo) are shown as boxes. Red triangles indicate loxP sites. Cleavage sites by HindIII, NotI, XbaI and XhoI are also shown. See METHODS for details. (B) DNA genotyping by PCR. (C) Real-time quantitative RT-PCR analysis of Ing2 mRNA expression in 8-week-old mouse testes. n = 3 per group. Ing2 expression levels (normalized to GAPDH) are shown on a scale of 10⁻⁴. (D) Western blot analysis of ING2 protein expression in mouse embryo fibroblasts. (TIF)

Figure S3 Histological analysis of testes from Ing2⁺/⁺ and Ing2⁻/⁻ mice at different ages. Testes (T) at 2 weeks to 24 months of age, as well as epididymis (E) at 24 months of age, were examined by H&E staining. Scale bars, 200 μm. (TIF)

Figure S4 Real-time qRT-PCR analysis of Asb4 and Gzmn mRNA expressions. The expression levels in Ing2⁻/⁻ testes are shown as the relative values to those in Ing2⁺/⁺ testes.
Abnormal spermatocytes were PUMA-positive in (white arrowheads). Scale bars, 100 μm. (TIF)

Figure S6 Immunohistochemical staining of PUMA protein. Tests sections from 8-week-old Ing2+/− and Ing2−/− mice were used. Spermatogonias with positive PUMA staining (black arrows) were observed in both Ing2+/− and Ing2−/− tests. Abnormal spermatocytes were PUMA-positive in Ing2−/− tests (white arrowheads). Scale bars, 100 μm. (TIF)

Figure S7 Kaplan-Meier survival curves of Ing2+/− (n = 22) and Ing2−/− (n = 28) mice. P = 0.43, Log-rank test. (TIF)

Figure S8 Representative images of histiocytic sarcoma of liver and lung in Ing2−/− mice. Scale bars are 100 μm (left panels) and 50 μm (right panels). (TIF)

Table S1 Selected genes differentially expressed in Ing2−/− mice. (DOC)

Table S2 Somatic cell-derived transcripts upregulated by trichostatin-A treatment were not significantly changed in Ing2−/− mice. (DOC)

Table S3 Histiocytic sarcoma arising in aging study. (DOC)

Table S4 Incidence of non-malignant lesions in aging study. (DOC)

Table S5 Degeneration of seminiferous tubules in aging study. (DOC)

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