Published in final edited form as:
*Cancer Res*. 2010 September 15; 70(18): 7264–7272. doi:10.1158/0008-5472.CAN-10-0820.

# Deficiency of Splicing Factor 1 suppresses occurrence of testicular germ cell tumors

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## Abstract

Testicular germ cell tumors (TGCTs) originate from germ cells. The 129-*Ter* and M19 (129.MOLF-Chr19 consomic) mouse strains have extremely high incidences of TGCTs. We found that the expression levels of *Sf1* encoded Splicing factor 1 (SF1) can modulate the incidence of TGCTs. We generated mice with inactivated *Sf1*. *Sf1* null mice (*Sf1*-/-) died before birth. Mice with one intact allele of *Sf1* (*Sf1*+/-) were viable but expressed reduced levels of *Sf1*. When *Sf1* deficient mice (*Sf1*+/-) were crossed to the 129-*Ter* and M19 strains, we observed decreased incidence of TGCTs in *Sf1*+/-;*Ter* and *Sf1*+/-;M19/+ mice compared to that in control cohorts. Therefore, *Sf1* deficiency protects against TGCT development in both strains. *Sf1* is expressed in the testes. We found that *Sf1* levels vary significantly in the testes of inbred strains such as 129 and MOLF and as such *Sf1* is an oncogenic tumor susceptibility factor from 129. Our results also highlight the complications involved in evaluating *Sf1* levels and TGCT incidences. When a large number of tumor promoting factors are present in a strain, the protective effect of lower *Sf1* levels is masked. However, when the dosage of tumor promoting factors is reduced, the protective effect of lower *Sf1* levels becomes apparent. SF1 is involved in splicing of specific pre-mRNAs in cells. Alternate splicing generates the complex proteosome in eukaryotic cells. Our data indicates that *Sf1* levels in mouse strains correlate with their incidences of TGCTs and implicate the importance of splicing mechanisms in germ cell tumorigenesis.

## Introduction

TGCTs are the most common malignancy in young men. These tumors originate from germ cells at different stages of development (1,2). Both genetic factors, such as ethnicity and family history, and environmental factors contribute to TGCT development (3,4). Evidence indicates that a combination of multiple genetic factors contribute to susceptibility to TGCT development (5-8). Individually, each of these factors contributes with relatively modest effects towards tumor development. It has been a challenge to identify the factors that cause TGCTs particularly because the tumors initiate *in utero* even though the disease may become evident decades after birth.

In mice, TGCTs occur predominantly on the 129 strain background. About 10% of 129 males develop spontaneous TGCTs (9). The genetic factors from the 129 strain that support TGCT development have not been identified. However, a number of gene defects have been experimentally shown to increase (10-14) or suppress TGCT incidences (15). The tumors in

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mice originate from primordial germ cells (PGCs) and initiate development around embryonic day (E) 11.5 - E13.5. For reasons not well understood, some PGCs on the 129 strain background become transformed to embryonal carcinoma (EC) cells. EC cells proliferate rapidly in the embryonic gonads. Soon after birth, EC cells differentiate randomly into embryonic and adult cells that constitute the TGCTs in the testes. TGCTs in mice resemble the pediatric TGCTs of humans (16).

Two 129 derived mouse strains, the 129-*Ter* and M19, have extremely high rates of spontaneous TGCT development (Supplementary Fig.1). The *Ter* defect is due to inactivation of the function of the RNA-binding protein, *Dead end 1* (*Dnd1*) (11). *Dnd1* is essential for PGC viability (11, 17). Loss of *Dnd1* results in progressive death of germ cells contributed to some extent by BAX-mediated apoptosis (18). This results in sterility in all *Ter* mice. However, 129 strain mice with inactivated *Dnd1* (129-*Ter* mice) develop TGCTs in addition to being sterile due to germ cell loss (19, 20). Thus, some PGCs of the 129-*Ter* strain escape death to transform into EC cells and EC cells subsequently differentiate to form large tumors in the testes.

A second mouse strain with high incidence of spontaneous germ cell tumors is the consomic, 129.MOLF-Chr19, mouse strain (also referred to as M19, chromosome substitution strain or CSS) (21). M19 strain differs from the 129 only because chromosome (Chr) 19 of the MOLF strain replaces that of the 129 (Supplementary Fig.1). The M19 strain does not carry the *Ter* (inactivation of *Dnd1*) defect. Multiple TGCT susceptible loci have been mapped to Chr 19 of the M19 strain. These loci either independently or interact epistatically to contribute to testicular tumor development (22). Unlike in the 129-*Ter* strain, the TGCT causing genes in M19 do not cause germ cell death. Thus both normal and transformed germ cells are present in the M19 strain and M19 males can be fertile despite having testicular tumors.

We identified *Splicing factor 1*, *Sf1*, as a TGCT candidate gene from the M19 strain (23). Here, we report the role of *Sf1* in TGCT development. Interestingly, our results indicate that *Sf1* expression levels influence the incidence of germ cell tumor development.

SF1 (also known as Splicing factor 1, Mammalian branch point-binding protein (mBBP), Zinc finger gene in MEN1 locus (ZFM1), Zinc finger protein 162 (ZNF162 or ZFP162)) participates in the early spliceosome assembly step during pre-mRNA splicing (24,25). SF1 is involved in the assembly of the earliest spliceosome complex (E' complex) committed to the splicing pathway (26,27). Splice site recognition requires cross talk between multiple proteins that are involved in forming complexes that commit the pre-mRNA to splicing. SF1 interacts co-operatively with U2 snRNP auxiliary factor ($U2AF^{65}$), and these proteins bind to the branch point site and the polypyrimidine tract in the intron of pre-mRNAs, respectively (28-30). SF1 is essential for viability of cells in culture. SF1 is not required for general splicing of all pre-mRNAs in cells but the premRNA substrates of SF1 that are necessary to maintain cell viability have not been identified (31). Thus, SF1 targets a sub-set of cellular pre-mRNAs for splicing and acts as an alternative splicing factor.

A previous study reported higher incidence of colon tumors when *Sf1*+/- mice were treated with an organotropic carcinogen (32). This indicated that lower levels of SF1 promote tumorigenesis. We report the results of genetic studies using *Sf1*+/- mice and contrary to the effects observed in the colon, we found that lower SF1 levels suppress germ cell tumor development. Therefore, our studies implicate SF1 and splicing mechanisms in germ cell tumor development.

## Materials and Methods

### Mouse strains

The 129.MOLF-Chr19 (21), 129 (129S1/SvImJ; JR002448, Jackson Laboratory, Bar Harbor, ME) and 129-*Ter* (11) strains have been described.

### Generation of Sf1 knockout mice

The gene trap 129/Ola strain embryonic stem (ES) cell clone, XD130, was purchased from BayGenomics (University of California, Davis). The trap vector insertion was validated by BayGenomics to contain gene trap inactivated *Sf1* by 5'-RACE (Rapid amplification of 5' complementary DNA ends) and sequencing which detected correct splicing between exon 1 of *Sf1* and the trap vector. XD130 was injected into C57BL/6J (B6) mice-derived blastocysts, which were then transferred into pseudopregnant recipient female mice and this yielded chimera mice. The chimeras were crossed to 129. Founder males which showed germline transmission of the gene-trap, β-*geo* allele, were crossed to 129 mice.

Germline transmission was screened by PCR genotyping for the targeted allele. Primers for genotyping were: gt1F, gt4R, v1F, v1R (Supplementary Fig.2). gt1F/i5R identify the wild-type allele. All animals were genotyped using the three sets of primers. Mice genotyped to be positive for both v1F/v1R and gt1F/gt4R were selected to maintain the *Sf1* gene trap line. Inactivation of *Sf1* by the β-*geo* encoding gene trap was further verified by Southern blotting and by sequencing of the genomic sequences flanking the vector and the vector insertion site.

### Southern blotting

Genomic DNA extracted from the spleen of 129.*Sf1*+/- and +/+ mice, of G1 and G2 generations, were digested with *Bgl* II or *EcoR* V and used for Southern blotting. Blots were hybridized with a $^{32}$P-labelled 626 bp *Pst* I/*Xba* I fragment derived from the β-*geo* cassette (Supplementary Fig.2). A 10.4 kb *Bgl* II fragment or a 8.9 kb *EcoR* V fragment is expected when one copy of trap vector is inserted in the genome. However, 8.6 kb and 10.4 kb *Bgl* II and 8.6 kb and 8.9 kb *EcoR* V fragments are expected when two copies of the trap vector are tandemly inserted into the *Sf1* gene.

*X-gal staining and in situ hybridization* was performed as described (33). *Immunohistochemistry and western blotting* for detection of SF1 used anti-SF1 antibody (SC-21157, Santa Cruz Biotechnology). Additional antibodies included β-actin (A5316 Sigma); anti-SSEA1-Alexa Fluor® 647 (SC-21702, Santa Cruz Biotechnology) and Sox9 (AB5535, Chemicon).

*RT-PCR and quantitative real-time PCR* was carried out as described (23). Primers used for qRT-PCR of SF1 were : 5'-ttcttcgttctgctgctttgc-3' and 5'-gtgaaaagaacgcgcattacac-3'; GAPDH: 5'-ttgtctcctgcgacttcaaca-3' and 5'-accaggaaatgagcttgacaaa-3'. Primers used for RT-PCR to amplify *Sf1* were: 5'-cacagcttcccacacccattaccc-3' and 5'-gctgctttgccgaaccatcc-3'; *Oct4*: 5'-ggaggaagccgacaacaatga-3' and 5'-tccacctcacacggttctcaa-3', *Dnd1*: 5'-gccctggtagaaggtcagtcac-3' and 5'-gccctgttcctaaacacttggtc-3'; *Sox9*: 5'-gcggagctcagcaagactctg-3' and 5'-atcggggtggtctttcttgtg-3'; *HPRT*: 5'-gttgagagatcatctccacc-3' and 5'-agctatgatgaaccaggtta-3'.

## Results

### *Sf1* is essential during embryonic development

We used a gene trap, ES cell clone XD130, to generate *Sf1*+/- mice. Southern blotting and PCR determined that XD130 harbored tandem insertion of two copies of the gene trap in the first intron of *Sf1* and 261 bp downstream of exon 1 (Supplementary Fig 2). This disrupts expression of normal *Sf1*. Instead, β-*geo* (fusion of *LacZ* encoding β-galactosidase and neo resistance gene) is fused with the first ten amino acids of *Sf1* and expressed from the targeted allele.

We intercrossed heterozygous *Sf1*+/- (or $Sf1^{\beta\text{-}geo/+}$) mice, but did not obtain any homozygous null mice, *Sf1*-/- ($Sf1^{\beta\text{-}geo\tilde{}\,\beta\text{-}geo}$), at weaning or at E11.5 (Supplementary Table 1). This result is similar to that reported for genetically modified *Sf1* mice made by Shitashige *et. al*. (32). Thus, *Sf1* is critical during embryonic development.

Next, we examined the levels of *Sf1* transcripts in *Sf1*+/- mice using quantitative RT-PCR (qRT-PCR). Total RNA from post-natal day 1 (PN 1) testes was used for qRTPCR. We found that *Sf1* mRNA levels were significantly lower (2 to 4-fold lower) in the testes of *Sf1*+/- mice compared to that in wild-type mice (Fig.1). SF1 protein levels had also decreased in PN 1 testes of *Sf1*+/- mice as determined by immunoblotting using anti-SF1 antibodies (Fig.1B and C).

### *Sf1* expression in the testes

We examined the expression pattern of *Sf1* in mouse testes. First, we examined PN 1 testes for LacZ activity from the endogenous *Sf1* promoter in *Sf1*+/- ($Sf1^{\beta\text{-}geo/+}$) mice. X-gal staining of *Sf1*+/- mice revealed that the germ cells were positive (Supplementary Fig.2) but the supporting cells of the seminiferous tubules were not positive for LacZ activity.

We sought to verify if the X-gal expression pattern in the testes of *Sf1*+/- mice was a true reflection of endogenous SF1 expression. We therefore performed *in situ* hybridization using antisense *Sf1* probes as well as immunohistochemistry using anti-SF1 antibody on PN 1 testes from normal, wild-type (+/+) mice. In contrast to the LacZ expression pattern, both *in situ* hybridization (Supplementary Fig.2) and immunostaining indicated (Fig.2) that SF1 is present in both the germ and Sertoli cells. Immunostaining indicated that SF1 was present in the nucleus as would be expected for a splicing factor.

Because we obtained contradictory data regarding LacZ expression as opposed to *in situ* hybridization and immunostaining, we used RT-PCR to determine whether *Sf1* transcripts are indeed expressed in both the germ and supporting cells. Therefore, we compared *Sf1* expression in the testes of *Ter* and wild-type mice. Mice homozygous for the *Ter* defect, and on a C57BL/6J background (B6-*Ter/Ter*), completely lack germ cells (11,19). RT-PCR indicated that *Sf1* was present in the testes of B6-*Ter/Ter* mice (Fig.2C). This indicates that *Sf1* transcripts are indeed present in the supporting cells of the testes. RT-PCR also indicated lack of expression of germ cell markers (*Dnd1* and *Oct4*) and presence of Sertoli cell markers (*Sox9*) in testes of *Ter* strain (18).

To determine why LacZ activity was absent in somatic cells, we examined lacZ transcript (*Sf1-geo* fusion transcript) in *Sf1*+/-;*Ter/Ter* testes. *Sf1*+/-;*Ter/Ter* mice also lack germ cells (strain described below). However, RT-PCR indicated that lacZ transcript is expressed in the somatic cells of the testes (Supplementary Fig. 2K). One reason we did not detect LacZ activity in the somatic cells of *Sf1*+/- testes could be because of lower levels of LacZ transcript and protein levels in the somatic cells. The LacZ reporter system is reported to be not as sensitive as RT-PCR or antibody staining (34).

Taking together the results of the above studies, we conclude that SF1 is expressed in both germ and Sertoli cells of the testes. However, in our *Sf1*+/- (*Sf1*$^{\beta\text{-}geo/+}$) mice, for reasons not known, lacZ activity is strong only in the germ cells.

## Deficiency of *Sf1* reduces TGCT incidence

Next, we examined the effect of *Sf1* deficiency on germ cell tumorigenesis. We were unable to assess TGCT incidence in *Sf1*-/- adult mice because of their embryonic lethality. We therefore crossed *Sf1*+/- mice to mouse strains with inherent genetic predisposition to TGCT. Males of the *Ter* strain develop testicular germ cell tumors at an exceptionally high rate. 94% of male mice homozygous for 129-*Ter* (*Ter/Ter*) develop tumor in at least one testes (19). In cases where testes of 129-*Ter* mice are tumor free, it remains small in size because of lack of germ cells.

We crossed *Sf1*+/- with *Ter*/+ mice and examined tumor incidence in the double mutant progeny (crosses are illustrated in Supplementary Fig.3). We found that 40% of *Sf1*+/-;*Ter/Ter* male mice have testicular tumors (Table 1). The tumor incidence of the control cohort of siblings of *Ter/Ter* genotype was 77%. Thus, tumor incidence had decreased in *Sf1*+/-;*Ter/Ter* mice and this change was statistically significant ($P$=0.0174). The incidence of bilateral testicular tumors had also decreased in *Sf1*+/-;*Ter/Ter* mice. 40% of *Sf1*+/-;*Ter/Ter* compared to 71% of *Ter/Ter* tumor bearing mice developed bilateral tumors. However, there was a corresponding increase in the number of *Sf1*+/-;*Ter/Ter* males with sterile, bilateral small testes (60%) compared to that in *Ter/Ter* males (23%). Mice of *Ter/Ter* genotypes with unilateral tumors had contralateral small testes. Thus, neither *Ter/Ter* nor *Sf1*+/-;*Ter/Ter* cohorts had normal testes.

Next, we examined *Sf1* levels in *Sf1*+/-;*Ter/Ter* testes. qRT-PCR indicated that *Sf1* transcript levels were lower in *Sf1*+/-;*Ter/Ter* compared to that in *Ter/Ter* testes (Supplementary Fig. 4). In addition, SF1 protein levels were also decreased in *Sf1*+/-;*Ter/Ter* testes (Fig.3A).

The incidence of tumors in *Ter/Ter* mice in this study was 77% and not the reported incidence of 94% (19). This could be because of the smaller sample size that was collected for this study (22 *Ter/Ter* and 25 *Sf1*+/-;*Ter/Ter* males were examined) (Table 1). An alternate possibility may be that 129 sub-strain differences influenced the tumor incidence. The *Sf1* gene trap was on 129/Ola sub-strain ES cells, founder mice were crossed to 129S1/SvImJ and *Ter* mice are another substrain, (129T1/Sv-+$^{p}$Tyr$^{c\text{-}ch}$*Ter*/+@*Na*) (35). This genetic variability between 129 substrains could have influenced the tumor incidences in *Ter/Ter* mice in our crosses.

Overall, although *Sf1* deficiency lowers the incidence of testicular tumors in *Ter/Ter* males, it does not restore germ cells in the testes of *Ter/Ter* males. This suggests that *Sf1* deficiency does not rescue the germ cell death in *Ter* (due to lack of DND1) but more likely affects germ cell transformation. Because SF1 mediates splicing of pre-mRNAs, it is likely that deficiency of SF1 causes reduced production of ‘oncogenic’ spliced variants, which leads to attenuation of germ cell transformation processes and an overall decreased level of germ cell tumors. This also suggests that SF1 functions downstream to DND1. The identities of the pre-mRNAs that are targets of SF1 in germ cells are at present unknown.

In conclusion, lack of one allele of *Sf1* results in decreased expression of SF1 and is correlated with a significant reduction of TGCT incidence in *Ter* mice. Thus, *Sf1* is haploinsufficient and *Sf1* levels influence germ cell transformation.

### Deficiency of *Sf1* reduces tumor incidence of M19 strain males

To determine how *Sf1* affects TGCT incidence of the M19 mouse strain, we crossed *Sf1*+/- to M19 mice and examined tumor incidences in the male progeny, *Sf1*+/-;M19/+ and M19/+ males (Supplementary Fig.3). These males carry one MOLF chromosome as signified by M19/+ and one allele of $Sf1^{\beta\text{-}geo}$ in *Sf1*+/-;M19/+. We found reduced TGCT incidence in *Sf1*+/-;M19/+ males (17%) compared to that in M19/+ males (27%) (Table 2). This reduction in tumor incidence was modest but statistically significant at $P < 0.04$. The incidence of bilateral tumors had also decreased in the *Sf1*+/-;M19/+ (18% bilateral tumors) compared to that in M19/+ males (31% bilateral tumors).

We found that SF1 levels had decreased, by about 50%, in *Sf1*+/-;M19/+ compared to the M19/+ strain (Fig.3A). This also correlated with lower *Sf1* levels in the *Sf1*+/-;M19/+ compared to that in M19/+ gonads and lower TGCT incidence in *Sf1*+/-;M19/+ mice. Next, we compared the *Sf1* mRNA levels in the PN1 testes of M19 to that of M19/+ and M19/+;*Sf1*+/- mice. The level of *Sf1* transcript was lower (by about 4-fold) in the M19 compared to that in the M19/+ gonads (Fig.3B). The M19/+ has one chromosome 19 from MOLF and one from 129 (Fig.4) whereas M19 is homozygous and both chromosome 19 are MOLF derived.

Overall, the genetic analysis using both the *Ter* and M19 strains indicates that lowering of *Sf1* levels in the testes protects against TGCT development. This indicates a common mechanism of action of *Sf1* in these two strains that develop TGCTs due to different genetic defects.

### *Sf1* levels in the 129 and MOLF inbred mouse strains

We had initially identified *Sf1* from the M19 strain and a comparative microarray screen had found a 2–8 reduced expression of *Sf1* in M19 compared to 129 (23). Because M19 males have higher TGCT incidence compared to 129 (21), we expected that decreasing *Sf1* levels further would increase TGCT incidence in *Sf1*+/-;*Ter* or *Sf1*+/-;M19 mice. Surprisingly, the results from our genetic data were opposite to that expected. We have verified that *Sf1* levels are indeed reduced in *Sf1*+/-, *Sf1*+/-;*Ter* and *Sf1*+/-;M19/+ mouse gonads. This raised the question as to why *Sf1* levels are low in the M19 strain, which has high tumor incidence. One explanation could be because Chr 19 of M19 strain is derived from and identical to that of the MOLF strain (Fig.4) (21). As *Sf1* is located on Chr 19, it is possible that the expression level of *Sf1* in M19 is inherited from MOLF.

Therefore, we examined *Sf1* expression of the MOLF inbred mouse strain. qRTPCR indicated that *Sf1* levels in the PN1 testes of MOLF are indeed similar to that in M19 (Fig. 3C) and *Sf1* levels in both MOLF and M19 is significantly lower than in the 129 strain. Therefore, MOLF has inherently low *Sf1* expression compared to 129. Also to note is the fact that, unlike the 129, the MOLF strain does not develop spontaneous TGCTs.

Thus, *Sf1* expression levels in M19 are similar to that in the MOLF strain although the testicular tumor frequency of M19 is extremely high and that of MOLF is extremely low.

Taking together all the above observations, we conclude that high testicular tumor incidence of M19 strain likely occurs in spite of the lower *Sf1* levels. Tumor development in M19 strain is due to multiple TGCT susceptibility loci from Chr 19 (22,23). The presence of multiple TGCT promoting genes likely overrides the protective effect of lower *Sf1* levels in M19. Thus the M19 strain is an exception to the rule that lower *Sf1* correlates with lower TGCT incidence.

However, when we reduce the dosage of the TGCT causing genes from that in M19 (homozygous for MOLF Chr 19) to M19/+ (heterozygous for MOLF Chr 19), the tumor incidence decreases in M19/+ to 26% (Fig.4). *Sf1* levels are higher in M19/+ (Fig.4). Only when we ***further lower*** *Sf1* levels, as in the *Sf1*+/-;M19/+ strain, both *Sf1* levels and tumor incidences decrease (Fig.4). Thus, the protective effect of lower *Sf1* levels becomes apparent when a single MOLF Chr 19 is present as in *Sf1*+/-;M19/+ mice.

## Discussion

Our results demonstrate that *Sf1* levels modulate the incidence of TGCT in two different mouse models of TGCTs. Haploinsufficiency of *Sf1* correlated with decreases in tumor incidences in *Sf1*+/-;*Ter/Ter* and *Sf1*+/-;M19/+ mice. This argues for a common role of SF1 in germ cell transformation in both strains considering that different genetic defects are responsible for germ cell tumorigenesis in the two strains. Reduction of *Sf1* levels likely reduces germ cell transformation rates and results in an overall reduced number of testicular tumors. Another possibility is that lower *Sf1* levels in germ cells may result in reduced viability of germ cells. Thus fewer germ cells survive to transform. However, PN1 testes of *Sf1*+/- mice did not appear to have fewer germ cells compared to wild-type mice and *Sf1*+/- mice are fertile.

*Sf1* levels in both MOLF and M19 strains were found to be significantly lower than in the 129 strain. Thus, different mouse inbred strains inherently express varying *Sf1* levels in their tissues and the *Sf1* levels could likely influence the tumorigenic potential of cells from these strains. Because the 129 strain has higher *Sf1* levels and is permissive for TGCT development, we propose that *Sf1* is an ‘oncogenic’ genetic susceptibility factor from 129 that promotes TGCT development.

Interestingly, *Sf1* levels are observed to inherently vary in tissues of different inbred strains (NCBI Geo Profiles). This inherent variation in *Sf1* levels could likely influence normal and disease phenotypes in mice.

We note that no significant tumor incidences have been observed for the MOLF inbred strain, which has inherently low *Sf1* levels. Of the reported studies, TGCT development is found in the M19 (129.MOLF Chr 19) consomic strain (21) and suppression of mammary tumorigenesis has been reported in FVB/N-Tg (MMTV-PyMT) and MOLF F1 hybrid mice (36).

The difference in SF1 levels between the 129 and MOLF strains may be due to a number of factors such as differences in the sequence and activity of the promoters between strains, differences in the nature of alternate spliced *Sf1* variants or SNPs (single nucleotide polymorphisms) in the transcripts that affect stability.

Contrary to other observations, we found that *Sf1* levels are low in M19 but TGCT incidence is high in this strain. In this case, it appears that the protective effect of low *Sf1* levels does not overcome the effects of multiple tumor promoting loci present in M19. However, when we reduce the dosage of tumor promoting loci in M19 to M19/+, then we clearly observe the protective effect of lower *Sf1* levels in M19/+;*Sf1*+/-. TGCT incidences correlate with *Sf1* levels in M19/+ and M19/+;*Sf1*+/- mice. Thus, the protective effect of *Sf1* deficiency can be overshadowed by the presence of multiple tumor promoting loci in the genome.

An earlier study reported a gene trap inactivated *Sf1* mouse line in which the gene trap was inserted in the promoter region of *Sf1* (37). Treatment of *Sf1*+/- mice with an organotropic carcinogen resulted in higher number of colon tumors (32). This indicated that lower *Sf1* levels are oncogenic and this is contradictory to our data. One explanation for this

discrepancy could be that *Sf1* functions as an oncogene or tumor-suppressor depending on the cell type. In colon cells, *Sf1* may be responsible for generating a majority of splice variants with tumor-suppressor function whereas in the testes, *Sf1* may generate mostly oncogenic splice variants. Thus lowering *Sf1* levels in the colon enhances tumorigenesis whereas lowering *Sf1* levels in the testes attenuates tumorigenesis. Another possibility is that application of DNA damaging carcinogen likely induces additional oncogenic mutations in colon cells, and the effects of multiple oncogenes outweigh the protective effect of *Sf1* deficiency. A third possibility is that because the *Sf1*+/- mouse backgrounds in the two studies are different, this influences the differences in results.

SF1 has been shown to be critical for HeLa cell viability (31). We detected SF1 expression in developmentally important organs, such as the heart and brain (Supplementary Fig.5). Presumably, there are cell-type specific pre-mRNA targets of SF1 that are critical for cell and embryonic viability.

In the testes, SF1 is found in both the germ cells and supporting cells of the seminiferous tubules. It is possible that SF1 function in the supporting Sertoli cells of the testes may also be important for germ cell tumorigenesis. Overall, alternative splicing events are most prevalent in the testis and brain (38), implying that splicing regulation is especially important in these tissues.

Transformation of cells frequently occurs in conjunction with dysregulation in alternative splicing (39-42). Because splicing factors usually regulate production of a variety of different mRNAs, changes in either the level or function of splicing factors can cause global changes in RNA levels and splicing variants. Specific splicing factors have been found to be overexpressed or downregulated in cancer tissues (42,43). Studies on human testicular tumors have found novel testicular cancer associated splice isoforms (44,45) which could serve as potential diagnostic or prognostic markers.

Interestingly, in humans, SF1 has also been implicated in immune responses (46). SF1 interacts with the branchpoint sequence within the intron of the histocompatibility leukocyte antigen, HLA-DQBβ. Naturally occurring and disease associated branchpoint mutations in DQβ1 intron 3 show impaired SF1 binding and altered splicing which influences their gene expression (46). A recent global analysis of the human transcriptosome revealed that many genes have subtle genetic changes, such as SNPs in splice site usage (47). Splicing differences due to SNPs were found to be frequent in human populations, affect disease-causing genes and likely contribute to phenotypic diversity and susceptibility to complex diseases.

In another study, oncogenic alternate splice variants were generated when SF1 was transiently transfected into human colon cancer cell lines and this correlated with cell transformation (37). This again indicates a proto-oncogenic capability of SF1 in human cells.

In summary, we provide genetic evidence showing that deficiency of SF1 suppresses testicular tumorigenesis. SF1, an RNA binding protein (48,49) necessary for spliceosome assembly of specific pre-mRNAs (24,25) likely regulates alternative splicing of pre-mRNAs in the testes. Thus, higher *Sf1* levels are oncogenic in the testes. Our data leads us to propose that SF1 is a tumor susceptibility factor for germ cell tumorigenesis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank Gilbert Cote for critical reading of the manuscript; Pierre McCrea and DongMin Gu for invaluable discussions. Funding for these studies were provided by CA75056 to JHN and CA093754 and Texas ARP to AM.

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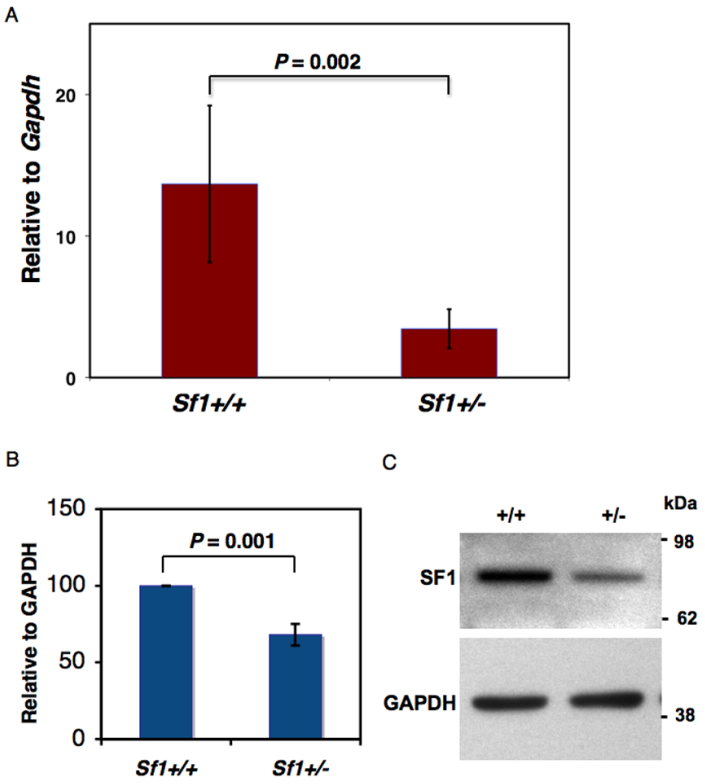


**Fig.1. Reduction of *Sf1* mRNA and protein levels in *Sf1*+/- mice**

(**A**) qRT-PCR performed on total RNA from PN1 testis of wild-type and *Sf1*+/- mice. *Sf1* expression is normalized against *Gapdh* expression in the testes. Error bar is the standard deviation derived from two independent experiments. (**B**) SF1 protein levels in PN1 testes of wild-type and *Sf1*+/- mice as determined by immunoblotting using anti-SF1 antibodies as shown in (C). Protein levels are normalized against GAPDH expression in the testes. Error bar is the standard deviation derived from four independent experiments. (**C**) A representative immunoblot using anti-SF1 antibody for PN1 testis of wild-type and *Sf1*+/- mice. The blots were re-probed with anti-GAPDH as controls.

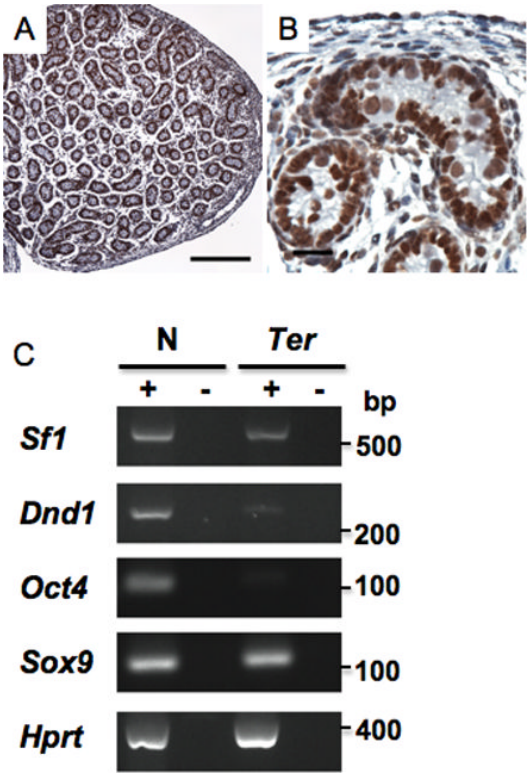


**Fig.2. Expression of *Sf1* in the testes**

(**A**) Immunostaining of PN1 testes from wild-type mice using anti-SF1 antibody at low (bar represents 200 um) and (**B**) higher magnifications (bar represents 20 um). (**C**) RT-PCR for *Sf1*, *Dnd1*, *Oct4*, *Sox9* and *Hprt* using total RNA from PN1 testes of wild-type (N) and B6-*Ter/Ter* (*Ter*) mice. PCR was performed on equal amounts of cDNA. + indicates presence of Superscript during cDNA preparation. - are control lanes and indicates no Superscript was added.

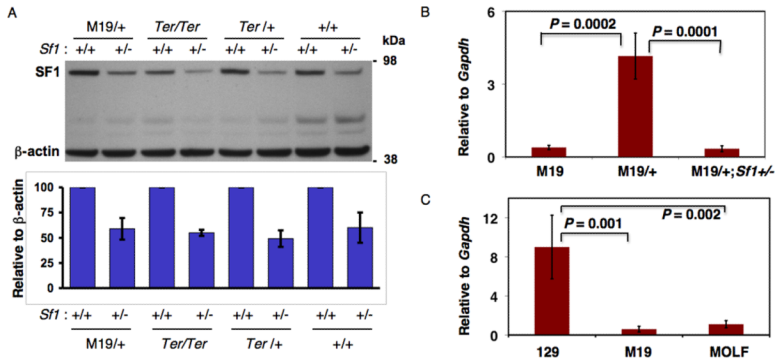


**Fig.3. Variation of *Sf1* levels in different mouse strains**

(**A**) (top) SF1 levels in *Sf1* deficient mice. PN1 testes, from M19/+, *Ter/Ter*, *Ter*/+ and wild-type (+/+) mice and corresponding *Sf1* deficient (*Sf1*: +/-) mice, were used for immunoblotting using anti-SF1 antibody. The blots were re-probed with anti-β-actin as controls. (bottom) Quantitation of SF1 levels in the mouse strains. The results are the average from 2 experiments. (**B**) qRT-PCR was used to determine *Sf1* levels in PN 1 testes of M19, M19/+ and M19/+;*Sf1*+/- strains. (**C**) Comparing *Sf1* levels in 129, M19 and MOLF strains. Total RNA from PN1 testes was used. The expression levels are normalized against *Gapdh* expression in the same sample. Error bar is the standard deviation derived from two independent experiments.

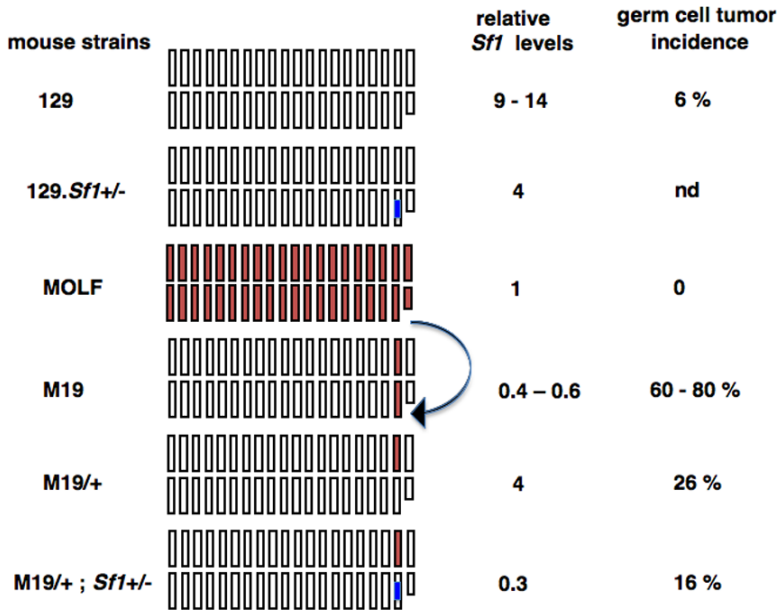


**Fig.4. Comparing *Sf1* levels and germ cell tumor incidences of different mouse strains**

The boxes represent the 19 autosomal Chrs and Chr X and Y (smaller box) of the mouse strains. White boxes represent 129 chromosomes. MOLF chromosomes are in red. The $Sf1^{\beta\text{-}geo}$ allele is represented by the blue mark on Chr 19. M19 strain carries Chr 19 of MOLF (indicated by arrow) and all other chromosomes are of 129 origin. Therefore, *Sf1* levels of M19 are similar to that in MOLF although tumor incidences of the two strains differ. M19/+ is heterozygous for MOLF Chr 19 and has higher levels of *Sf1*. Introduction of $Sf1^{\beta\text{-}geo}$ allele to generate M19/+;*Sf1*+/- mice results in lowering of *Sf1* levels and concomitant reduction of tumors compared to that in M19/+. The relative *Sf1* levels are the median values from the graphs in Figs.1 and 3.

**Table 1**

***Sf1* deficiency reduces incidence of germ cell tumors. TGCT incidence in *Ter* mice deficient for *Sf1***

*Ter/+; Sf1+/-* mice were intercrossed with *Ter/+* mice (illustrated in Supplementary Fig.3). Progeny were of genotypes listed in the table. Progeny were genotyped and the testes phenotypes examined. The significance of the difference in tumor incidences between the *Ter/Ter* and *Ter/Ter;Sf1+/-* strains is $P = 0.0174$. Differences in the tumor incidences of *Ter/+* compared to *Ter/+;Sf1+/-* and +/+ compared to *Sf1+/-* were not statistically significantly.

| Genotype | No. with tumors | Bilateral tumors | Unilateral tumors | Sterile testes | Normal testes | No. of males examined |
|---|---|---|---|---|---|---|
| *Ter/Ter* | 17 (77%) | 12 (71%) | 5 | 5 (23%) | 0 | 22 |
| *Ter/Ter; Sf1+/-* | 10 (40%) | 4 (40%) | 6 | 15 (60%) | 0 | 25 |
| *Ter/+* | 23 (45%) | 5 | 18 | 0 | 28 | 51 |
| *Ter/+; Sf1+/-* | 21 (40%) | 4 | 17 | 0 | 32 | 53 |
| +/+ | 0 | 0 | 0 | 0 | 20 | 20 |
| *+/+; Sf1+/-* | 1 (5%) | 0 | 1 | 0 | 21 | 22 |

**Table 2**

**TGCT incidence in M19 mice deficient for *Sf1***

M19 (or M19/M19) was crossed to *Sf1*+/- mice (Supplementary Fig.3). The progeny were genotyped to determine the M19/+ and M19/+;*Sf1*+/- males. Testes phenotypes of all the males were examined. The significance of the difference in tumor incidences between the strains is $P = 0.0375$.

| Genotype | No. with tumors | Bilateral tumors | Unilateral tumors | Normal testes | Total number of males examined |
|---|---|---|---|---|---|
| *M19/+* | 36 (27 %) | 11 (31%) | 25 | 95 | 131 |
| *M19/+;Sf1+/-* | 22 (17%) | 4 (18%) | 18 | 111 | 133 |