Loss of the Transmembrane but not the Soluble Kit Ligand Isoform Increases Testicular Germ Cell Tumor Susceptibility in Mice

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Abstract

Several genetic variants act as modifiers of testicular germ cell tumor (TGCT) susceptibility in the 129/Sv mouse model of human pediatric TGCTs. One such modifier, the Steel locus, encodes the transmembrane-bound and soluble ligand of the kit receptor. Some (*Sl* and *Sl^J^*) but not all (*Sld*) mutations of the Steel locus increase TGCT incidence in heterozygous mutant mice. Because *Sl* and *Sl^J^* are large deletions that affect multiple transcripts and *Sld* is an intragenic deletion of the kit ligand (*Kitl*) from which only the soluble protein is produced, it was uncertain whether *Kitl* or a neighboring gene is a modifier of TGCT susceptibility. We tested the effect of the small *Steel grizzle-belly* (*Slgb*) deletion on TGCT susceptibility to determine whether *Kitl* is a TGCT modifier gene. An increase in TGCT incidence was observed in *Slgb*/+ heterozygotes, and fine mapping of the deletion breakpoints revealed that *Kitl* is the only conventional gene deleted by the mutation, suggesting that *Kitl* is the TGCT modifier gene at the Steel locus. Additionally, we propose that soluble KITL in *Sld*/+ heterozygous mutant mice complements a dosage effect of transmembrane-associated kit ligand on TGCT susceptibility and that the kit receptor (*Kit*) is haplosufficient for primordial germ cell development. [Cancer Res 2008;68(13):5193–7]

Introduction

Testicular germ cell tumors (TGCT) are the most common cancer affecting young men (1). Although both environmental and genetic factors contribute to TGCT susceptibility, the genetic component of TGCTs is particularly strong. Statistically, 25% of susceptibility to early-onset TGCTs is attributable to genetic factors, making these tumors the third most heritable form of cancer (2). Despite the strong heritable influence on TGCT incidence and considerable effort to identify susceptibility genes, the genetic basis for TGCTs remains elusive. A recent study concluded that many loci with weak effects control TGCT susceptibility, and to date, only one genetic factor, the rare *gr/gr* Y chromosome deletion, is reproducibly associated with TGCT incidence in humans (3–5).

In mice, spontaneous TGCTs occur at an appreciable frequency only on the 129 inbred background (6). TGCTs in mice are most similar to human pediatric TGCTs and arise during embryogenesis as a result of abnormalities in the development of the primordial germ cell (PGC) lineage, the embryonic precursor of oocytes, and sperm (7, 8). As in humans, the genetics of TGCT susceptibility is complex with as many as six to eight segregating genes controlling susceptibility in mouse crosses (6, 9). In segregating crosses between 129 and other inbred strains, the frequency of affected males is <0.01% (9). Thus, TGCT susceptibility in mice is too complex for conventional genetic approaches.

Modifier genes are an attractive alternative to the limited power of conventional genetic approaches to dissect the genetic basis of highly complex traits. Several single gene mutations and genetic variants modify TGCT susceptibility when congenic on the 129/Sv background (10). Such modifiers have been useful in defining genetic interactions that control TGCT susceptibility and identifying susceptibility loci in sensitized polygenic trait analyses (11–14).

The *Steel* (*Sl*) and *White-spotted* (*W*) variants are a group of classic spontaneous mouse mutations affecting the kit ligand (KITL) and its receptor (KIT), respectively (15–19). The KITL/KIT system promotes the survival, proliferation, and migration of hematopoietic, melanocyte, and germ cell precursors (20). In homozygous *Sl* and *W* mice, defects in the development of these progenitor cells causes anemia, loss of pigmentation, and infertility (20). In addition, many homozygous *Sl* and *W* embryos die after embryonic day 15.5 (E15.5; ref. 18). Heterozygotes for most of the *Sl* and *W* mutations present less severe phenotypes than homozygotes (15). To test whether mutations in the KITL/KIT system increase TGCT susceptibility, various *Sl* and *W* mutations were transferred to the 129/Sv background and TGCT incidence was measured in heterozygous mutants and their wild-type siblings (6, 9). Interestingly, some (*Sl* and *Sl^J^*) but not all (*Sld*) Steel mutations, and none of the tested *W* mutations, increase TGCT susceptibility in heterozygotes (Table 1).

Variation among the *Sl* and *W* alleles was initially puzzling because it was difficult to understand how increased TGCT susceptibility could be limited to only two variants of this ligand-receptor complex. Discovery of the molecular nature of the *Steel* mutations provided clues. *Sl* and *Sl^J^* are large deletions (~973 and ~650 kb, respectively) that remove all of *Kitl* and a significant amount of flanking sequence (Fig. 1*A*; refs. 18, 21). By contrast, *Sld* is a 4-kb intragenic deletion of *Kitl* that retains expression of soluble but not transmembrane KITL at wild-type levels (Fig. 1*A*; refs. 22, 23). Therefore, two explanations are possible for increased TGCT incidence in *Sl* and *Sl^J^* but not *Sld* mutant mice: (*a*) a gene or noncoding regulatory element neighboring *Kitl* is also deleted in *Sl* and *Sl^J^* mutants and is responsible for increased TGCT susceptibility, or (*b*) gene dosage effects of soluble *Kitl* on TGCT susceptibility reduces TGCT incidence to 129/Sv levels in *Sld* mutants.

The *Steel grizzle-belly* (*Slgb*) mutation is a smaller deletion (~120 Kb) than *Sl* or *Sl^J^*, with the 5′ breakpoint ~60 to 40 Kb proximal to exon 1 of *Kitl* and the distal breakpoint within the 3′ untranslated region of *Kitl* (Fig. 1*A*; ref. 18). Preliminary sequence analysis of the *Slgb* deletion map suggested that *Kitl* may be the only gene deleted by this mutation. Therefore, the *Slgb* mutation provides a means to test the influence of a *Kitl* null deletion on TGCT susceptibility independent of alterations in other genes.

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Table 1. TGCT incidence in *Kitl* (*Sl*) and *Kit* (*W*) heterozygous mutant mice

Mutation	Sample size*	Percentage of mice with at least one TGCT	
		Wild-type (+/+)	Mutant (M/+)
Sl/+	280	2.0	14.0
*Sl*J/+	2,389	2.5	8.9
*Sl*d/+	1,614	5.0	5.0
W/+	650	4.0	2.0
W3J/+	122	2.0	0
Wx/+	232	2.0	0
Wv/+	896	2.0	3.0

NOTE: L.C. Stevens and D.S. Varnum (*Sl*/+ data, personal communication; ref. 6).
*The relative numbers of wild-type and mutant mice were not published or available for these crosses. Therefore, statistical significance was not calculated.

In this report, we tested the influence of heterozygosity for the *Slgb* mutation on spontaneous TGCT incidence in 129/Sv males and fine mapped the *Slgb* deletion breakpoints. An increase in TGCT incidence was observed in *Slgb*/+ heterozygotes. Mapping of the *Slgb* deletion breakpoints revealed no other genes or obvious functional elements within the deletion interval, suggesting that partial deficiency of *Kitl* increases TGCT susceptibility. We propose that soluble KITL maintains TGCT susceptibility at 129/Sv baseline frequencies in *Sld*/+ heterozygous mutant mice and that KIT is haplosufficient for PGC development in mice with the *W* mutations.

Materials and Methods

Mice. Mice (129S1/SvImJ; JR002448) were obtained from the Jackson Laboratory. Heterozygous *Kitl*^{Slgb}/+ mutant mice were obtained from the research colony of M.A. Bedell (Department of Genetics, University of Georgia, Athens, Georgia; ref 24, 25). Before the tumor survey, the *Kitl*^{Slgb} mutation was backcrossed for at least 10 generations onto 129S1/SvImJ to establish a 129S1/SvImJ-*Kitl*^{Slgb}/+ (*Slgb*/+) congenic strain. Homozygous *Slgb/Slgb* mutant embryos were obtained from timed matings of heterozygous mutant mice. Embryonic day 0.5 (E0.5) was assumed to be noon of the day the vaginal plug was observed, and embryos were then collected at E13.5. Homozygous *Slgb/Slgb* embryos die after E15.5 (18). Mice were maintained in the Case Western Reserve University Animal Resource Center on a 12:12-h light:dark cycle and fed Lab Diet 5010. All protocols were approved by the Institutional Animal Care and Use Committee.

Genotyping. A light coat color on the belly and pink tips of the tail and digits on an otherwise normally pigmented mouse distinguished *Slgb*/+ mice from their wild-type siblings (12, 15). Genomic DNA was isolated from adult tails and E13.5 embryo tissue using the Qiagen DNeasy Blood and Tissue kit. Homozygous mutant embryos were identified using a PCR assay for *Kitl*. After a 5-min incubation at 95°C, samples were amplified for 35 cycles: 30 s at 95°C, 30 s at 58°C, and 1 min at 72°C, followed by a final extension at 72°C for 5 min. Primers were as follows: forward 5′-TCATGGTGCACCGTATCCTA-3′ and reverse 5′-CCTTGGCATGTTCTTC-CACT-3′ and produce a 170-bp product from wild-type +/+, and *Slgb*/+ DNA but not homozygous *Slgb/Slgb* DNA. Sequence information was obtained from the University of California Santa Cruz (UCSC) Genome Browser using National Center for Biotechnology Information (NCBI) Mouse Build 37.[1]

***Slgb* tumor survey and statistics.** Crosses between 129/Sv and 129/Sv–*KitlSl*^{gb}/+, in both parental directions, were used for the TGCT surveys. Male offspring were necropsied at ages 4 to 6 wk, and testes were visually examined for tumors. χ^2 contingency tests were used to test for statistical differences between the number of affected wild-type and *Slgb*/+ males, and parent-of-origin effects on TGCT susceptibility.

***Slgb* deletion mapping.** The distal *Slgb* breakpoint was previously identified to be within the 3′ UTR at bp position 5,287 of the *Kitl* transcript (18, 26). To identify the proximal breakpoint of the *Slgb* deletions, we used a genotyping and sequencing strategy (21). Primer sets were designed to PCR amplify sequences 60, 50, 40, and 30 kb proximal to the start of *Kitl*. A PCR product was generated for all primer sets using wild-type adult DNA, but homozygous *Slgb/Slgb* mutant embryo DNA failed to generate a product with the 30-kb primer set, placing the proximal breakpoint between −40 and −30 kb of *Kitl*. New primer sets were designed at 1-kb intervals between −40 and −30 kb of *Kitl*, and an additional round of PCR further narrowed the region of the proximal breakpoint to between −38.3 and −37.2 Kb. PCR was then performed on *Slgb/Slgb* homozygous and wild-type DNA with primers 5′-CACAGGTTAAAATATGCACAGCA-3′ (forward; upstream of the 5′ breakpoint) and 5′-ATCAAAAGGGTCGGGACATA-3′ (reverse; downstream of the 3′ breakpoint) to amplify across the deletion. A 463-bp product was generated from only the *Slgb/Slgb* homozygous DNA. Sequencing of this PCR product defined the boundaries of the *Slgb* deletion.

Sequence analysis. Sequence analysis of the Ensembl,[2] NCBI,[3] and UCSC mouse genome databases was used to identify transcripts and hypothetical proteins within the deletion intervals of the Steel mutations. The Vista human/mouse/rat Genome Browser[4] and the UCSC Genome Browser Vertebrate Multiz Alignment & Conservation Track were used to identify conserved extragenic sequences within the *Slgb* deletion interval (27, 28).

Results

***Slgb* increases the frequency of affected males in 129/Sv mice.** If *Slgb* (~120 kb deletion) increases the number of affected males as do *Sl* and *Sl*J (~973 kb and ~650 Kb deletions, respectively; Table 1), the candidate region that controls TGCT susceptibility in Steel mutant mice would be significantly reduced. We therefore measured the TGCT incidence in *Slgb/+* mutant and wild-type male siblings. TGCT incidence was significantly increased (~2-fold) in *Slgb*/+ mutant males compared with wild-type controls (Table 2). Therefore, our *Slgb*/+ tumor survey reduces the critical interval for TGCT susceptibility in Steel mutant mice to ~120 Kb.

Additionally, we tested the influence of the parent-of-origin of the *Slgb* mutation on TGCT incidence in Slgb/+ and wild-type offspring. Parental factors did not affect susceptibility in *Slgb*/+ offspring as TGCT incidence was similar irrespective of the parental origin of the *Slgb* mutation (Table 3). By contrast, the parent-of-origin of the *Slgb* mutation influenced TGCT susceptibility in wild-type offspring, with TGCT incidence being significantly lower when the male parent was heterozygous for *Slgb* (Table 3). A more detailed analysis of this effect will be reported elsewhere.

Haploinsufficiency of KITL. We mapped and sequenced the breakpoints of the *Slgb* deletion to identify candidate TGCT susceptibility genes. The *Sl* mutation spans several genes, including *Kitl*, and several predicted functional elements, including two putative Ensembl miRNAs distal to *Kitl* and several hypothetical

[1] http://genome.ucsc.edu
[2] http://www.ensembl.org
[3] http://www.ncbi.nlm.nih.gov/genome/guide/mouse
[4] http://pipeline.lbl.gov

proteins (Fig. 1*A*; ref. 21). The *Sl*^{J} mutation also deletes the two putative Ensembl miRNAs and several hypothetical proteins (Fig. 1*A*; ref. 18). Additionally, the proximity of the deletion breakpoints to neighboring genes may affect their expression. Based on the rough map of the ~120 Kb *Slgb* deletion, it seemed that *Kitl* is the only gene affected by the mutation (Fig. 1*A*; ref. 18).

To fine-map the breakpoints for the *Slgb* mutation, we used a combination of PCR and sequencing. The *Slgb* deletion breakpoints were mapped to bp 99,440,172 and 99,563,881 of mouse chromosome 10 (Fig. 1*B*). This 123-Kb deletion starts 38 Kb proximal to exon 1 of *Kitl* and ends within its 3′UTR. Sequence analysis did not reveal any other genes, transcripts, or expressed sequence tags within the *Slgb* interval in the mouse or other mammalian species. The closest annotated sequences to the proximal end of the *Slgb* deletion are a hypothetical protein, a ribosomal protein L17–like pseudo-gene (XM_001480394), and *citrate synthase–like protein* (*Csl*), −112 Kb and −220 kb from the *Slgb* breakpoint, respectively.

The VISTA program and UCSC genome browser revealed several cross-species conserved extragenic sequences within the 38 Kb proximal of *Kitl*. However, due to the distance between the closest neighboring gene and the proximal breakpoint of the *Slgb* deletion, the cross-species conserved extragenic elements within the *Slgb* deletion are most likely *Kitl* transcriptional regulatory elements. In addition, because the *Slgb* distal breakpoint ends within the *Kitl* 3′UTR, the mutation most likely does not affect the hypothetical proteins or the putative Ensembl miRNAs distal to *Kitl*. Furthermore, the known miRNAs on mouse chromosome 10 do not associate with tumor susceptibility loci on this chromosome (29). Together our TGCT survey and sequence analysis of the *Slgb* mutation suggest that deficiency of *Kitl* increases TGCT susceptibility in mice.

Discussion

The KITL isoforms differentially influence TGCT susceptibility in mice. An increase in TGCT incidence was observed in 129/Sv mice harboring the *Slgb* deletion of the Steel locus. Furthermore, sequencing of the *Slgb* deletion breakpoints revealed that *Kitl* is the only conventional gene deleted by this mutation. These results suggest that *Kitl* is the TGCT modifier at the Steel locus. Interestingly, only the KITL null deletions (*Sl*, *Sl*^{J}, and *Slgb*) influence TGCT susceptibility on the 129/Sv inbred background. The *Sl*^{d} mutation, from which soluble KITL is expressed, does not influence TGCT susceptibility. The contrasting effects of the various Steel mutations on TGCT susceptibility suggest that the transmembrane isoform of KITL has unusual and specific effects on PGC development.

Both KITL isoforms are biologically active but may have distinct functional significance in the survival (transmembrane KITL),

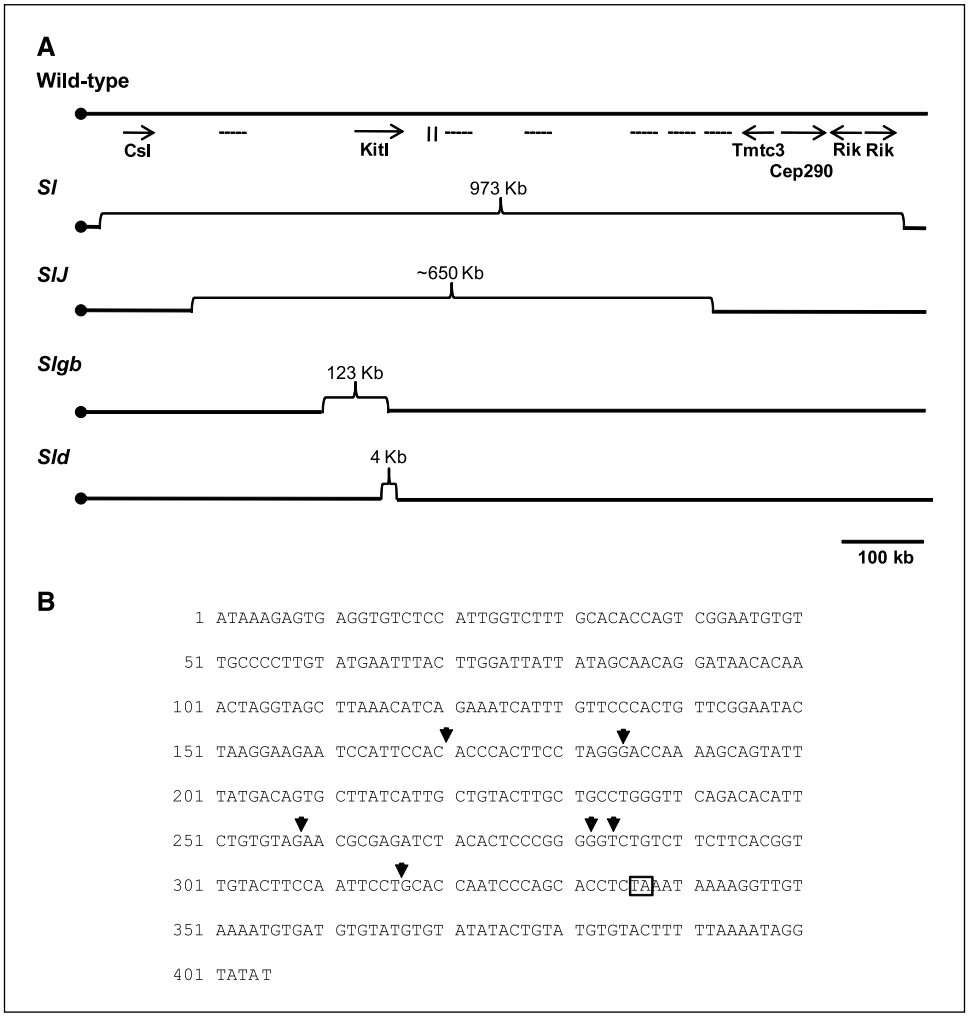

Figure 1. Mapping of the *Slgb* deletion. *A,* comparison and extent of several mutations of the Steel locus. Schematics of murine chromosome 10 of wild-type, *Steel* (*Sl*), *Steel J* (*Sl*^{J}), *Slgb*, and *Steel Dickie* (*Sl*^{d}) alleles are shown with the centromere (●) on the left and the telomere on the right. The size of each deletion is indicated in kilobases. The exact breakpoints for *Sl*^{J} are unknown. *Arrows,* known genes [*Csl, kit ligand* (*Kitl*), *transmembrane and tetratricopeptide repeat* (*Tmtc3*), and *centrosomal protein Cep290* (*Cep290*)] and two RIKEN clones (4930430F08Rik and 1700017N19Rik); *vertical lines,* two putative Ensembl microRNAs (ENSMUSG00000077043 and ENSMUSG00000077034); *dashed lines,* locations of several hypothetical proteins. *B,* fine mapping of the *Slgb* deletion breakpoints. A 463-bp PCR product that spans the *Slgb* deletion was sequenced. Boxed nucleotides indicate the position of the 5′ and 3′ breakpoints (chromosome 10 bp 99,440,172 and 99,562,881, respectively, of NCBI Mouse Build 37), with 122,708 bp of genomic sequence deleted between these two breakpoints. Alignment of the *Slgb* deletion sequence with the C57BL/6J reference sequence identified six single nucleotide polymorphisms (*SNP*).

Table 2. Increased incidence of spontaneous TGCTs in grizzle-belly (*Slgb*) males

Genotype	No. of males	No. of affected males	Frequency of affected males	Test score (χ^2, *P*)
Slgb/+	229	21	9%	5.8, <0.025
Wild-type	260	10	4%	

proliferation (transmembrane and soluble KITL), and migration (soluble KITL) of stem cells (30–32). Deficiency of KITL in homozygous and heterozygous Steel null mutants results in PGC proliferation, migration, and survival defects (18, 21, 25). By contrast, in homozygous *Sld/Sld* and compound heterozygous *Sld/Sl* mice, which are transmembrane KITL-deficient, expression of soluble KITL from the *Sld* locus partially rescues germ cell proliferation and migration (25, 31). Furthermore, PGC development (and TGCT susceptibility) in *Sld*/+ mice is similar to wild-type siblings. Thus, although soluble KITL cannot fully complement the functions of transmembrane KITL, it seems to compensate for the dosage effect of transmembrane-bound KITL on PGC development and TGCT susceptibility in *Sld*/+ heterozygous mice.

Parent-of-origin effects of the *Slgb* mutation on wild-type TGCT incidence. The parent-of-origin effect of the *Slgb* mutation on wild-type tumor incidence in our *Slgb* crosses was unexpected. Recently, we showed that genetic modifiers of TGCTs interact across generations to influence TGCT susceptibility (transgenerational epistasis; ref. 13). These interactions represent a novel mode of epigenetic inheritance and may account for the difficulties in identifying TGCT susceptibility genes in mice and humans. Influences of parental genotype on wild-type TGCT incidence have not, however, been previously reported for single genetic modifiers. These parental effects have either not been tested, reported, or do not occur for most TGCT modifiers. We are currently in the process of replicating the *Slf* tumor survey to determine whether the parent-of-origin effect observed in the *Slgb* survey is a common feature of Steel null mutations.

KIT haplosufficiency. Most of the pleiotropic effects observed in Steel homozygous and heterozygous mutant mice are also observed in mice with the various *W* mutations of the KIT receptor. However, unlike the Steel mutations, none of the tested *W* mutations increased TGCT susceptibility in heterozygous males. PGC development in heterozygous *Sl* and *W* mutation mice may explain this difference. Heterozygous Steel mutant mice have reduced numbers of PGCs, due to migration and proliferation deficiencies, and a defect in spermatogenesis in the adult testis (15, 21, 33). By contrast, heterozygous *W* mutant mice have a statistically similar number of PGCs in the developing genital ridge and type A spermatogonia in the adult testis compared with wild-type littermates (34, 35). Therefore dosage of *Kitl* but not *Kit* affects PGC development. Because TGCTs initiate during embryogenesis, it is therefore not surprising that heterozygosity for *W* mutations does not modify TGCT susceptibility.

Abnormalities in PGC development and TGCT susceptibility. The mechanism by which the Steel loss-of-function mutations increase TGCT susceptibility is unknown. It is possible that the PGC migration defect of Steel mutant mice contributes to TGCT formation. Transmembrane-bound KITL is expressed by somatic cells along the route of PGC migration and the genital ridge releases chemoattractants, such as soluble KITL, to guide PGCs toward the developing gonad (30, 36–38). When migratory signals are disrupted and male PGCs localize to ectopic tissues, they undergo abnormal developmental changes and initiate apoptosis in response to local stimuli (39, 40). It is possible that in heterozygous null mutant males, disruption of KITL signaling delays germ cell migration into the genital ridge, exposes PGCs to signals outside the developing gonad, and enhance tumorigenic potential after localization into the embryonic testis.

Alternatively, PGCs with altered migration patterns may become apoptotic before entering the genital ridge, resulting in decreased PGC numbers within the developing testis, as is observed in Steel mutant mice (15, 21, 33). A decreased ratio of germ cells to somatic cells within the developing testis may increase the availability of growth factors produced by the somatic cells and alter the developmental fate of the PGCs that localized to the genital ridge. Because Steel mutations act as modifiers of a preexisting susceptibility to TGCTs in 129/Sv inbred mice, it is likely that the effects of delayed migration or altered germ cell to somatic cell ratios are specific to the 129 genetic background.

KIT mutations in human TGCTs. *KIT* is one of several candidate TGCT susceptibility genes located within a region of human chromosome 4 (4q12) that is frequently amplified in seminomas but not nonseminomas (41, 42). Somatic mutations that constitutively activate the KIT receptor are also associated with seminoma initiation but are extremely rare in nonseminomas and

Table 3. Parent-of-origin effect revealed by *Slgb* reciprocal crosses

Genotype	No. of males	No. of affected males	Frequency of affected males	Test Score (χ^2, *P*)
Paternal *Slgb Slgb*/+	84	7	8%	0.11, NS
Maternal *Slgb Slgb*/+	145	14	10%	
Paternal *Slgb* wild-type	98	0	0%	6.29, <0.025
Maternal *Slgb* wild-type	162	10	6%	

Abbreviation: NS, not significant.

pediatric teratomas (42–45). By contrast, deletions of chromosome 12q22, where *Kitl* is located, are associated with nonseminomas but are extremely rare in seminomas (46, 47). In one series of studies, up to 40% of TGCTs showed loss of heterozygosity for a polymorphic marker (D12S7) closely associated with *Kitl* (47, 48). Interestingly, 4q12 amplifications, 12q22 deletions, and activating *Kit* mutations are rare in TGCT precursor lesions, intratubular germ cell neoplasia (ITGCN; refs. 41, 49). Therefore, modulation of the KIT/KITL signaling cascade is most likely not involved in TGCT initiation but, instead, influences the ultimate developmental fate of ITGCN. Because mouse TGCTs are teratomas and teratocarcinomas, our data and those from humans suggest that a decrease in KIT/KITL signaling, through a gene dosage affect of *Kitl*, supports development of differentiated tumors (teratomas or nonseminomas), whereas activation of KIT/KITL signaling supports development of less differentiated seminomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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