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BAX-mediated cell death affects early germ cell loss and incidence of testicular teratomas in *Dnd1^{Ter/Ter}* mice

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Abstract

A homozygous nonsense mutation (*Ter*) in murine *Dnd1* (*Dnd1^{Ter/Ter}*) results in a significant early loss of primordial germ cells (PGCs) prior to colonization of the gonad in both sexes and all genetic backgrounds tested. The same mutation also leads to testicular teratomas only on the 129Sv/J background. Male mutants on other genetic backgrounds ultimately lose all PGCs with no incidence of teratoma formation. It is not clear how these PGCs are lost or what factors directly control the strain-specific phenotype variation. To determine the mechanism underlying early PGC loss we crossed *Dnd1^{Ter/Ter}* embryos to a *Bax*-null background and found that germ cells were partially rescued. Surprisingly, on a mixed genetic background, rescued male germ cells also generated fully developed teratomas at a high rate. Double-mutant females on a mixed background did not develop teratomas, but were fertile and produced viable off-spring. However, when *Dnd1^{Ter/Ter}* XX germ cells developed in a testicular environment they gave rise to the same neoplastic clusters as mutant XY germ cells in a testis. We conclude that BAX-mediated apoptosis plays a role in early germ cell loss and protects from testicular teratoma formation on a mixed genetic background.

Keywords

Dnd1; *Ter*; testicular teratoma; teratocarcinoma; testicular germ cell tumor

Introduction

Testicular teratomas arise as benign, non-seminomatous germ cell tumors characterized by the differentiation of a diverse array of cell and tissue types within the tumor, including cartilage, muscle, hair, and glandular tissue. Although spontaneous testicular teratomas are virtually non-existent in mice, Leroy Stevens established a substrain of mice in the 1950s (129Sv/J) in which the incidence of testicular teratomas was approximately 1% (Stevens and Little, 1954; Stevens 1959). In 1973, Stevens discovered a spontaneous mutation on the 129Sv/J genetic background (called *Ter*) that raised the incidence of teratomas to 17% in heterozygotes and 94% in homozygous mutants, but never induced ovarian teratomas (Noguchi and Noguchi, 1985; Stevens, 1973). Despite the higher incidence on the 129Sv/J background, when *Ter* was crossed

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onto other inbred strains, such as C57BL/6J, LTXBJ, and C3H/HeJ, testicular teratomas were not found (Noguchi et al., 1996; Stevens, 1981). The variable penetrance of the tumor phenotype in *Ter* mice of different genetic backgrounds faithfully mimics the etiology of germ cell tumors in humans. Germ cell tumors are the most common form of cancer in men between the ages of 15 and 34, and are highly correlated with ethnicity and other complex genetic influences (Brown et al., 1987; Brown et al., 1986; Hussain et al., 2008; Linger et al., 2007).

The identity of the *Ter* locus was recently mapped to a gene called *Dead-end 1 (Dnd1)*, which is expressed in primordial germ cells (PGCs) (Youngren et al., 2005). Although teratomas in 129Sv/J *Ter (Dnd1^{Ter/Ter})* mice are male-specific, both sexes show a severe, but incomplete, loss of PGCs early in development on all genetic backgrounds. It is not known how PGCs are lost in *Dnd1^{Ter/Ter}* mutants, or how strain-specific differences contribute to teratoma formation. In zebrafish, where the *Dnd* phenotype was originally characterized, PGCs are specified but never exhibit motile characteristics, and thus fail to migrate to the site of the developing gonad and are lost completely (Weidinger et al., 2003). No tumor formation has been reported in zebrafish *Dnd* morphants. In mice, although the normal number of PGCs is specified, there is an immediate decline in germ cell population size in mutant embryos compared to wild-type, beginning at the time of migration (around E8.5) (Sakurai et al., 1995). However, unlike the zebrafish phenotype, no migration defect was detected. By the time germ cells have arrived in the gonad (E11.5), wild-type germ cells number in the thousands while only a few dozen (at most) persist in the *Dnd1^{Ter/Ter}* gonad. On most genetic backgrounds, adult *Dnd1^{Ter/Ter}* males are completely sterile, with no germ cells remaining and no evidence of teratomas after birth. By contrast, in the 129Sv/J testis, germ cells are either lost or give rise to teratomas in ~95% of homozygous mutants during fetal development (Stevens, 1973). Female *Dnd1^{Ter/Ter}* mice on the 129Sv/J background (and all other backgrounds tested) are subfertile but show no incidence of teratoma formation indicating that either the XX germ cell or the ovarian environment is not susceptible to this pathway of teratoma formation.

Elegant genetic studies have identified several loci on particular 129 chromosomes that act as modifiers of the tumor phenotype (Hammond et al., 2007; Heaney et al., 2008; Heaney and Nadeau, 2008; Lam et al., 2007; Lam and Nadeau, 2003). The mechanism of how these loci interact with *Dnd1* on a molecular level has yet to be determined. Because teratoma formation likely involves both cell autonomous and paracrine signaling, it is important to determine whether *Dnd1* expression is restricted to germ cells in the developing gonad, or also extends to the somatic cells that surround germ cells and regulate their development. *In situ* expression studies reveal *Dnd1* transcripts in the genital ridge of developing male and female gonads. In E12.5–E14.5 XY gonads, the expression pattern is limited to testis cords, which contain both Sertoli cells and germ cells (Youngren et al., 2005). One group has shown that the transformed Sertoli cell lines TM4, 15P-1, and MSC1 do not express DND1 (Bhattacharya et al., 2007). However, a conflicting report demonstrated that *Dnd1^{Ter/Ter}* mutant gonadal somatic cells failed to support cultures of wild-type or mutant germ cells, suggesting a secreted *Ter*-dependent factor necessary for germ cell survival (Takabayashi et al., 2001).

To resolve these issues and investigate strain- and sex-specific phenotypes, we have taken genetic and cell biology approaches. We demonstrate that *Dnd1* is expressed only in germ cells in the developing testis between E12.5 and E15.5. We found that XX germ cells in the context of a testis can also initiate teratoma formation. By genetically blocking BAX-mediated apoptosis we show that early germ cell loss in mutants is at least partially due to apoptosis. Blocking cell death in mutants on a mixed genetic background is also associated with an increased incidence of testicular teratomas. Our data support a model where BAX-mediated cell death plays a role in the initial loss of mutant germ cells and suggest that the rate of transformation and perhaps the efficiency of cell death pathways differ between 129Sv/J and other strains.

Materials and Methods

Mice, timed matings, and genotyping

Dnd1^{Ter/+} mice were provided by Dr. Joseph Nadeau at Case Western Reserve University and maintained on the 129Sv/J background. *Bax^{+/-}* mice (1-Baxtm1Sjk/J B6.129) were imported from Jackson Laboratories and maintained on a mixed genetic background (129Sv/J;C57BL/6J). *Dnd1^{Ter/+}* mice were crossed to *Bax^{+/-}* mice and the F1 offspring were intercrossed to maintain the line and obtain double heterozygous animals for timed matings. *Sox9-ECFP* mice were maintained as a homozygous line on a C57BL/6 background and outcrossed to CD-1 for timed matings. XY *Sry^{MYC}* mice were maintained on a 129Sv/J strain, crossed to *Dnd1^{Ter/+}* mice to obtain heterozygotes, and the progeny were mated with *Dnd1^{Ter/+}* females. For timed matings, males and females were setup in the afternoon and the next morning females were checked for plugs and counted as day E0.5 if positive. Tail DNA was extracted using standard methods and genotyped using the following primer sets: TerF 5'-GTA GTT CAG GAA CTC CAC TTG TG-3', TerR 5'-GCT CAA GTT CAG TAC GCA C-3', Bax ex5F 5'-GAG CTG ATC AGA ACC ATC ATG-3', Bax In5R 5'-GTT GAC CAG AGT GGC GTA GG-3', Bax NeoR 5'-CCG CTT CCA TTG CTC AGC GG-3', MycF 5'-AAT CAT AGC AAG GGG GAG TGT TG-3', MycR 5'-ATG GAG AGC TTG GGC GAC CTC-3'. *Bax* mice were genotyped using the protocol found on the Jax website. *Sry^{MYC}* mice were genotyped by PCR using an annealing temperature of 60°C and running the 130bp product on a 2% agarose gel. *Dnd1^{Ter}* mice were genotyped by PCR using an annealing temperature of 62°C. The PCR product (145bp) was digested overnight at 37°C with the restriction enzyme DdeI and run on a 4% agarose gel or 6% acrylamide gel. DdeI digestion of DNA from mice with the *Dnd1^{Ter}* mutation produces 123bp and 22bp products.

Flow cytometry and RT-PCR

E12.5–E15.5 *Sox9-ECFP* gonads were dissected and separated from the mesonephros, collected on ice in PBS, and trypsinized at 37°C for 15 minutes. The trypsin was removed and 500uL of Dulbecco's Modified Eagle Medium was added to stop the reaction. The tissue was drawn through a 27 ½ gauge needle to break up cells, then put over a cell strainer and spun down briefly to collect the flow through. The suspension was subjected to fluorescence activated cell sorting (FACS) at a core facility. ECFP-positive and negative cell fractions were obtained in PBS and centrifuged to pellet the cells. The PBS was aspirated and a total RNA extraction was performed using TRIzol® (Invitrogen) and chlorophorm with a 100% isopropanol precipitation and 75% ethanol wash. cDNA was generated from RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. PCR was performed on each cDNA fraction using primers for *Dnd1*, *Sox9*, *Oct4*, and *HPRT* using an annealing temperature of 62°C for 29 cycles. Each primer set was designed to span an intron and one primer from each set to overlap an exon-exon boundary by at least 7-nucleotides on each side. Primers used for each are as follows: Dnd1F 5'-GCC CTG GTA GAA GGT CAG TCA C-3', Dnd1R 5'-GCC CTG TTC CTA AAC ACT TGG TC-3', Sox9F 5'-GCG GAG CTC AGC AAG ACT CTG-3', Sox9R 5'-ATC GGG GTG GTC TTT CTT GTG-3', Oct4F 5'-GGA GGA AGC CGA CAA CAA TGA-3', Oct4R 5'-TCC ACC TCA CAC GGT TCT CAA-3', HprtF 5'-TGG ACT GAT TAT GGA CAG GAC TGA A-3', HprtR 5'-TCC AGC AGG TCA GCA AAG AAC T-3'. The products were run on a 2% agarose gel and imaged using a Bio-Rad gel-doc and Quantity One software imaging system.

In situ hybridization

Gonads were dissected, fixed in 4% paraformaldehyde (PFA) overnight at 4°C, washed in 0.1% Tween-20/PBS, dehydrated in 100% methanol and stored at -20°C until used. Full-length *Dnd1*, cloned into pGEM®-T Easy vector (Promega) was used as a probe. In situ hybridization on gonads was performed as described previously (Henrique et al., 1995; Kim et al., 2006). A

digoxigenin labeled RNA probe for *Dnd1* was detected using an alkaline phosphatase-conjugated anti-digoxigenin antibody.

Busulfan treatment

CD-1 pregnant females were given a 150 mg/kg (~4.5 mg/mouse) dose of Busulfan. 30 mg Busulfan was dissolved in 500 μ L 95°C DMSO and combined with an equal volume of 95°C water, then cooled to 37°C before intraperitoneal injection at E10.5. Mice were dissected at E14.5 and germ cell depletion was confirmed by alkaline phosphatase staining of one pair of gonads from the litter. The remainder were processed for *in situ* hybridization.

Immunofluorescence and histology

Fluorescent immunocytochemistry was performed on whole mount or cryosectioned gonads which were fixed overnight at 4°C in 4% PFA, washed in PBS for whole mount antibody staining, or put through a sucrose gradient (10%, 15%, 20%, 20%:OCT 1:1 overnight at 4°C) before embedding in OCT for sectioning. Antibodies (Rat anti-GCNA1 (kindly provided by George Enders 1:50), Rabbit anti-MVH (Abcam cat. #ab13840 1:500), Rat anti-E-cadherin (Zymed Laboratories cat. #13-1900 1:500)) were added to the blocking solution (PBS and 0.1% Triton X-100, 3% BSA, and 10% heat-inactivated goat serum) and incubated rocking overnight at 4°C (or stationary for sections). Samples were washed four times over a 30 minute period in washing solution (PBS and 0.1% Triton X-100, 3% BSA, and 1% heat-inactivated goats serum), and then incubated 1 hour at room temperature in blocking solution with Cy2-, Cy3-, or Cy5-conjugated secondary antibodies (1:500; Jackson Immunoresearch). Samples were then washed four times for 30 minutes total in washing solution, then mounted in DABCO and imaged on a Zeiss LSM510 confocal microscope. For histology, ovaries were dissected and fixed in Bouin's solution overnight, then washed thoroughly in 70% ethanol. After embedding in paraffin, ovaries were sectioned (5 μ m), stained with haematoxylin and eosin, and mounted in DABCO.

Results

Dnd1 is expressed only in germ cells of the developing testis, not in Sertoli cells, between E12.5 and E15.5

Previous experiments indicated that somatic cells may be altered in *Dnd1*^{Ter/Ter} testes (Takabayashi et al., 2001). To determine whether *Dnd1* expression is restricted to germ cells or is also characteristic of Sertoli cells, we injected a chemotherapeutic agent, busulfan, into pregnant females to eliminate germ cells from the developing embryonic gonads by E14.5. We then compared the *Dnd1* expression pattern to that of uninjected E14.5 testes (Fig. 1A,C). After busulfan treatment, expression of *Dnd1* was eliminated in the testis (Fig. 1B,D), suggesting that *Dnd1* is either not expressed in Sertoli cells or requires the presence of germ cells.

To investigate this question further we made use of the transgenic reporter line, *Sox9-ECFP*. The *Sox9-ECFP* line contains an ECFP reporter driven by a *Sox9* enhancer element (Sekido and Lovell-Badge, 2008), and is specific to Sertoli cells in the gonad. RT-PCR analysis of sorted cells between stages E12.5 and E15.5 revealed *Dnd1* expression in the ECFP-negative pool that contains *Oct4* expressing cells (germ cells), but not in the ECFP-positive (Sertoli cell only) pool (Fig. 1E,F). Note that cell sorting did not eliminate all *Sox9*-positive cells from the negative pool (e.g., *Sox9*-positive cells remain in the *Sox9-ECFP*-negative pool). However, the *Sox9-ECFP*-positive pool is pure and contains no *Oct4*-expressing cells (Fig. 1E). These experiments indicate that *Dnd1* transcript is located within germ cells and not in Sertoli cells.

Early germ cell loss in mutants is partially due to BAX-mediated apoptosis

Dnd1^{Ter/Ter} embryos suffer a significant reduction in the number of germ cells arriving in the gonads of both sexes on all backgrounds tested (Sakurai et al., 1995). However, the fate of male and female mutant germ cells diverges after colonization of the gonad. The few mutant XX germ cells that successfully migrate to the gonad are maintained in the ovary and give rise to mature oocytes, albeit in greatly reduced numbers. Adult mutant females have small ovaries and are sub-fertile, but can produce offspring at low rates when crossed with wild-type males (Noguchi and Noguchi, 1985). By contrast, mutant XY germ cells colonize the testis but transform on the 129Sv/J background, giving rise to testicular teratomas at a rate of approximately 95% (Fig. 2A,B). On other backgrounds, XY germ cells are lost completely by perinatal stages. Examination of a mixed background *Dnd1^{Ter/Ter}* testis revealed that by postnatal day eleven (P11), no germ cells could be detected by analysis with antibodies against mouse Vasa homolog (MVH) or germ cell nuclear antigen 1 (GCNA1) (Fig. 2C–D).

It is unknown why this drastic phenotypic variation occurs between strain backgrounds. We initially hypothesized that the tumor susceptibility phenotype could be positively correlated with the number of germ cells colonizing the gonads in *Dnd1^{Ter/Ter}* embryos. Previously, alkaline phosphatase stains had been carried out on mutant C57BL/6J gonads to identify and count PGC numbers (Sakurai et al., 1995). However, upon examination of PGC numbers in 129Sv/J mutants it was obvious that there was no significant difference in germ cell population size in mutant gonads between these different backgrounds (data not shown). This indicates that the mechanism underlying early loss of germ cells is similar to both backgrounds and suggests that the strain-specific tumor phenotype is not affected by the number of colonizing germ cells, but rather by other strain-specific properties.

The early PGC loss common to mutants of all backgrounds is not well-understood. Although the correct number of PGCs is specified in *Dnd1^{Ter/Ter}* mice, as early as E8.5, a difference can be noted in the number of migrating germ cells entering the hindgut between mutants and wild-type, based on alkaline phosphatase staining (Sakurai et al., 1995). Germ cell loss is exacerbated during migration, as only a few dozen germ cells reach the *Dnd1^{Ter/Ter}* gonad (compare Fig. 3A and 3C). However, reports have been inconclusive in determining whether differences in mutant germ cell number are due to active cell death, lack of proliferation, or differentiation during migration. We investigated whether cell death plays a role in early germ cell loss by genetically blocking one apoptotic pathway.

Based on data showing that blocking BAX-mediated apoptosis rescues PGCs in other germ cell-deficient mutants (Stallock et al., 2003; Suzuki et al., 2008), we crossed the *Bax* mutation (1-Bax^{tm1Sjk/J} B6.129) onto *Dnd1^{Ter}* mice. To this end, *Bax* heterozygotes on a mixed genetic background (129Sv/J. C57BL/6J. CD1) were mated to *Dnd1* heterozygotes of the 129Sv/J background to obtain double heterozygotes. These offspring were intercrossed to produce compound double-mutants on a mixed background. *Dnd1^{Ter/Ter};Bax^{-/-}* double-mutants showed a significant germ cell rescue (~50%) by stage E13.5 on a mixed background and in both sexes (Fig. 3G and data not shown), indicating that *Bax*-mediated apoptosis is an important mechanism of initial germ cell loss in *Dnd1^{Ter/Ter}* mutants. However, the rescued phenotype is not complete indicating that there may be other BAX-independent cell death pathways involved, defects in proliferation, or aberrant differentiation contributing to the loss of mutant germ cells.

Dnd1^{Ter/Ter};Bax^{-/-} males develop testicular teratomas on a mixed genetic background

In *Dnd1^{Ter/Ter}* males, few germ cells colonize the gonad, but those that do either transform on a 129Sv/J genetic background or undergo cell death resulting in testes completely devoid of germ cells by birth on other genetic backgrounds. We investigated whether rescuing early germ

cell loss on a *Bax* mutant background would lead to the formation of teratomas in a non-129Sv/J testis with increased numbers of mutant germ cells. Surprisingly, on a mixed genetic background, we found that double-mutant male samples exhibited typical testicular teratomas (Fig. 3H) at a high rate, as compared to single *Dnd1^{Ter/Ter}* mutant testes (Fig. 3D) on a similar background, which are normally devoid of germ cells by the time of birth. However, the incidence of teratoma formation in mice on a mixed background (Table 1) was slightly lower than that reported for a pure 129Sv/J background, possibly due to a smaller sample size: 91% (20/22) of mixed background double-mutants developed teratomas, compared to ~95% on the 129Sv/J background. The double-mutants that did not develop teratomas lost their germ cells entirely. Moreover, 52% (12/23) of *Dnd1^{Ter/Ter};Bax^{+/-}* mice on a mixed background developed teratomas, although smaller in size and typically unilateral. As a control, *Dnd1^{Ter/Ter};Bax^{+/+}* samples on this mixed background were examined and none (0/10) developed teratomas by P20. At this stage, all XY gonads from this group were completely devoid of germ cells.

These experiments suggest that while mutant germ cells on the 129Sv/J background efficiently generate teratomas, mutant germ cells on other backgrounds also have this capacity, but are less efficient at undergoing the transformation event, or are normally cleared more efficiently by competing mechanisms of cell death.

***Dnd1^{Ter/Ter};Bax^{-/-}* females do not form teratomas and are fertile, but XX germ cells in a testis can transition into neoplastic clusters**

In *Dnd1^{Ter/Ter}* mutants, the significant early loss of germ cells results in sub-fertile females (Noguchi and Noguchi, 1985). The few germ cells that colonize the gonad survive throughout ovarian development. However, the ovaries are small and contain very few oocytes (Fig. 4A). Rescuing the early germ cell loss on a *Bax* mutant background resulted in double-mutant females that maintained many more oocytes than *Dnd1^{Ter/Ter}* single mutants alone, as determined by histological analysis (Fig. 4A–B). Additionally, these double-mutant females were fertile and produced viable, fertile offspring when out-crossed to CD-1 mice (data not shown). As reported previously, there was no incidence of ovarian teratoma in mutant females (Noguchi et al., 1996).

The fact that testes and not ovaries form teratomas in *Dnd1^{Ter/Ter}* embryos indicates that there is something specific either to XY germ cells or to the male pathway of development that predisposes mutant germ cells to transformation. To distinguish between these possibilities, we crossed 129Sv/J XY *Sry^{MYC}* males to 129Sv/J *Dnd1^{Ter/+}* heterozygous females. *Sry^{MYC}* mice contain an autosomal MYC-tagged SRY protein that causes female-to-male sex reversal. Intercrosses between XX *Dnd1^{Ter/+}* heterozygotes and XY *Sry^{MYC}; Dnd1^{Ter/+}* heterozygotes produced sex-reversed XX *Dnd1^{Ter/Ter}* testes. Normally, mutant XY germ cells on a 129Sv/J background give rise to transforming germ cell clusters during embryonic development. These clusters can be identified histologically as early neoplasias that ultimately give rise to the nascent teratomas (Cook et al., in preparation; Rivers and Hamilton, 1986). We are able to detect these clusters based on morphology and immunofluorescent staining for E-cadherin by E18.5 (Fig. 5C arrow and inset). Examination of mutant XX germ cells in a sex-reversed testis at this stage also revealed similar neoplasias (Fig. 5D arrow and inset). This indicates that the initiation of teratoma formation is dependent upon a male developmental pathway and not the sex chromosomes of the PGCs. Despite the presence of XX neoplasias (n=2/3 at E18.5) that resembled the early stage of teratoma formation in XY testicular germ cells, we never saw mature teratomas after birth in XX *Sry^{MYC}; Dnd1^{Ter/Ter}* mice (n=0/10 at P14 and later). Few germ cells persist in sex-reversed controls compared to normal testis controls at later developmental stages and after birth (data not shown). As XX germ cells are usually lost perinatally in XX sex-reversed males (McLaren, 1984), it is possible that germ cell loss and

other testis cord defects reported for XX sex-reversed testes (Ishii et al., 2007) prevent formation of fully-developed teratomas even though early neoplasias can be identified.

Despite a role in germ cell death, loss of *Dnd1* does not affect embryonic viability

While only germ cells express *Dnd1* in the developing gonad, *in situ* analysis detects transcript in other tissues of the developing embryo (Youngren et al., 2005) and ESTs have been identified in different organs of the adult including the heart and brain. Since loss of *Dnd1* in PGCs can lead to BAX-mediated apoptosis, it is possible that loss of expression in other tissues could also lead to cell death. Corroborating this, a previous report observed a role for DND1 in embryonic viability (Bhattacharya et al., 2007). It was shown that loss of *Dnd1* on a 129Sv/J background, but not on a C57BL/6J background, leads to embryonic lethality.

We examined five hundred progeny from *Dnd1*^{Ter/+} 129Sv/J crosses and calculated the ratios of wild-type, heterozygous, and homozygous mutants (Table 2). We found that the proportions fell within Mendelian ratios, with a P-value greater than 0.975. These data indicate that, in our colony on a 129Sv/J genetic background, the *Dnd1*^{Ter} mutation does not affect embryonic viability.

Discussion

Dnd1 expression

Testicular teratomas in *Dnd1*^{Ter/Ter} mice are believed to arise from fetal germ cells that are misregulated in the developing gonad. We have shown here that *Dnd1* is expressed in germ cells and not in Sertoli cells between E12.5–15.5, the time period when teratoma formation begins in *Dnd1*^{Ter/Ter} mutants (Rivers and Hamilton, 1986). This expression pattern indicates that the direct effects of the *Dnd1*^{Ter} mutation occur in germ cells, and not in the somatic supporting cell lineage. Teratomas arise from defects in the intrinsic cellular program in germ cells, within the context of the strain-specific background. It is not yet clear whether the genetic background affects the frequency or degree of misregulation of the intrinsic germ cell program, or whether it affects the somatic environment and its ability to regulate or clear mutant germ cells.

While expression of *Dnd1* is important for germ cell development, little is known about its function in other tissues. ESTs and other expression analyses indicate that *Dnd1* is expressed in various organs such as heart and brain, suggesting other possible roles for *Dnd1* in development and adult life. Here we investigated five hundred adult mice from heterozygous crosses in our colony and found no evidence for a negative impact of the *Dnd1*^{Ter} mutation on embryonic viability on a 129Sv/J genetic background. The difference between our finding and a previous report demonstrating a role for *Dnd1* in embryonic viability on the 129Sv/J background (Bhattacharya et al., 2007) could be due to sub-strain differences that have arisen since separation of the two colonies.

Early cell death

Dnd1^{Ter/Ter} mice display two very distinct phenotypes: (1) initial loss of germ cells common to both sexes on all genetic backgrounds and (2) male-specific teratoma formation only on the 129Sv/J genetic background. The germ cell loss phenotype is similar to the *Nanos3* null phenotype (Suzuki et al., 2008). Like DND1, NANOS3 is a conserved RNA binding protein expressed in PGCs after specification. A null mutation in *Nanos3* leads to the loss of a high proportion of germ cells prior to their arrival in the gonad, similar to *Dnd1* mutants. Also like the *Nanos3* mutants, blocking BAX-mediated apoptosis only partially rescues the germ cell loss. This suggests that other mechanisms may contribute to germ cell loss in both mutants, including BAX-independent cell death, lack of proliferation, or even trans-differentiation of

germ cells during migration. While it was found that the *Nanos3* null germ cells did not trans-differentiate or have proliferation defects during migration (Suzuki et al., 2008), this has not been investigated in *Dnd1* mutants.

Zebrafish DND binds the 3'UTR of *Nanos* in primordial germ cells to promote translation by protecting the transcript from miRNA-mediated repression (Kedde et al., 2007). Based on the high conservation of *Nanos* in organisms from *C. elegans* to *H. sapiens*, it is possible that a similar mechanism of early misregulation of the murine *Nanos3* transcript (functionally homologous to zebrafish *Nanos*) leads to the early germ cell loss phenotype of *Dnd1*^{Ter/Ter} mutants (Kedde and Agami, 2008; Saga, 2008).

Testicular teratoma formation and the role of BAX

Bax rescue significantly increases the number of oocytes in adult females, which makes them more fertile than *Dnd1*^{Ter/Ter} single mutant ovaries. These females produce viable, fertile offspring, indicating that PGCs rescued in *Bax* mutants are not significantly impaired, at least with respect to the female developmental pathway. This suggests that teratomas arise during the male developmental program as the result of a secondary function of DND1 within germ cells in the fetal testis. This model is further supported by our results, which demonstrate that the genotypic sex of the germ cells is irrelevant to tumor susceptibility. Rather, the testis environment is crucial, as XX *Dnd1*^{Ter/Ter} germ cells developing within a testis are also susceptible to transformation.

The 129Sv/J specific phenotype of testicular teratoma formation in *Dnd1*^{Ter/Ter} mice has been particularly enigmatic since the early 1970s. Very few germ cells colonize the gonad, sometimes none, but those that arrive frequently transition into teratomas, or are lost by birth. Previously we hypothesized that the number of colonizing germ cells might be higher in 129Sv/J mice and could be directly related to the incidence of teratomas. However, there is virtually no difference in initial colonizing germ cell numbers between strain backgrounds. Therefore this does not explain the inherent strain difference in susceptibility.

Nonetheless, we found that if the number of mutant germ cells in the *Dnd1*^{Ter/Ter} testis is increased by blocking BAX-mediated apoptosis, teratomas arise on a mixed genetic background at a rate of 91% and at a rate of 52% in *Bax* heterozygotes, even though heterozygotes do not show an increase in germ cell number. Two explanations for these results seem plausible: (1) an increased number of germ cells due to specific loss of the *Bax* pathway may compromise the capacity of the non-129Sv/J fetal testis to clear misregulated germ cells; and/or (2) the transition of mutant germ cells to teratoma occurs at a lower frequency in backgrounds other than 129Sv/J, and inhibiting apoptosis increases the number of germ cells and uncovers this lower frequency event.

The rate of tumor formation on the mixed *Dnd1*^{Ter/Ter};*Bax*^{-/-} background is quite high and intriguingly, tumors also arise in *Bax* heterozygotes (although smaller and typically unilateral). This suggests that BAX may normally eliminate mutant germ cells from the fetal testis prior to their transition into teratomas. However, BAX-mediated apoptosis is obviously not the only mechanism for clearing defective germ cells, as mutant germ cells are still cleared in some double homozygous mutants (2/22 double-mutant samples without teratomas in Table 1). This reasoning might predict that pathways for clearing mutant germ cells, including the BAX pathway, are less efficient in the 129Sv/J strain, which could explain the high incidence of teratomas. To investigate the hypothesis that *Bax* is expressed at lower levels in 129Sv/J gonads than in other strains we performed quantitative RT-PCR (qRT-PCR) on E13.5 gonads from 129Sv/J and C57BL/6J strains and found that *Bax* transcript levels between backgrounds are virtually identical (data not shown). These data do not rule out the possibility that *Bax* activity differs between strains. However, the reported frequency of teratoma formation is slightly

higher in 129Sv/J *Dnd1^{Ter/Ter}* mutants (95%) than in *Dnd1^{Ter/Ter};Bax^{-/-}* mutants on a mixed genetic background (91%), which may suggest an additional factor that affects the rate of transformation and penetrance of the phenotype. It remains to be seen whether similar tumor susceptibility results will be uncovered for double mutants on a pure non-129Sv/J background (C57BL/6J for example). We are in the process of back-crossing the *Bax*-null allele to a pure C57BL/6J background for an analysis of germ cell rescue and teratoma formation.

More research is necessary to fully understand the effects of *Bax* rescue and the strain-specific differences inducing testicular teratomas. To elucidate the underlying cause of transformation in *Dnd1^{Ter/Ter}* germ cells, it will be essential to perform a full molecular analysis of the transition of mutant germ cells into nascent teratomas. Importantly, the *Dnd1^{Ter/Ter};Bax^{-/-}* double-mutant is a tool that can be used to compare mutant germ cells undergoing transformation to those that are not. A critical event that occurs during the period of male germ cell development when teratomas arise is mitotic arrest. Mitotic arrest is presumably not occurring in mutant germ cells based on the large size of the tumors that form and previous findings that proliferation is not arrested in *Dnd1^{Ter/Ter}* mutants (Rivers and Hamilton, 1986). Among targets of DND1 recently identified in human cell lines are several cell cycle regulators that are involved in mitotic arrest in the fetal testis (Kedde et al., 2007; Western et al., 2008). Future studies will determine whether misregulation of mitotic arrest plays a direct role in tumorigenesis or aberrant development of germ cells in *Dnd1^{Ter/Ter}* mice. Linking the cell cycle to cellular pluripotency in developing germ cells will undoubtedly shed light on pathways involved in tumorigenesis. Investigating these unusual tumors which reflect the close relationship between germ cells, embryonic stem cells, and tumor cells, will reveal important clues about the mechanisms that program these cell fates and regulate the transitions between them.

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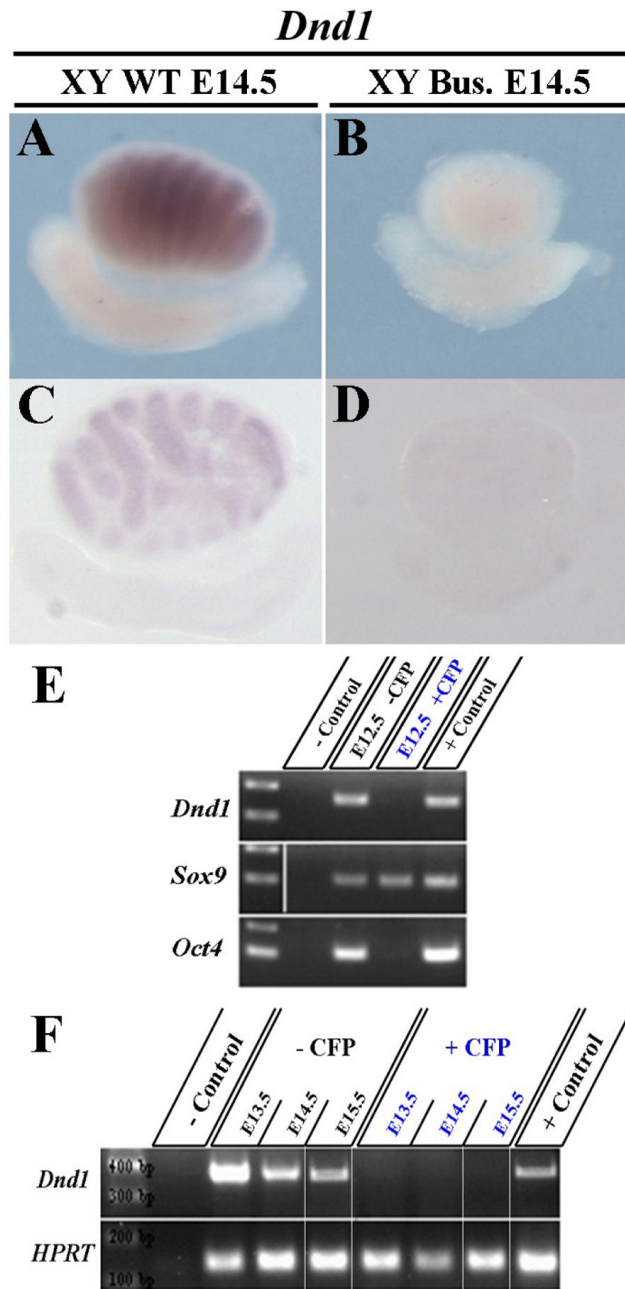


Figure 1. Expression of *Dnd1* is restricted to germ cells of the developing testis
Dnd1 *in situ* expression in a whole mount (A,B) and sectioned (C,D) E14.5 WT testis is restricted to testis cords, and disappears in a Busulfan treated testis (B,D). Busulfan is a chemical that eliminates germ cells. (E,F) RT-PCR on *Sox9-ECFP* positive and negative cells, sorted from E12.5–E15.5 gonads. *Dnd1* expression is only detected in the ECFP-negative fraction (containing the *Oct4* positive germ cell population) and is excluded from the pure Sertoli cell fractions (exclusively *Sox9* positive).

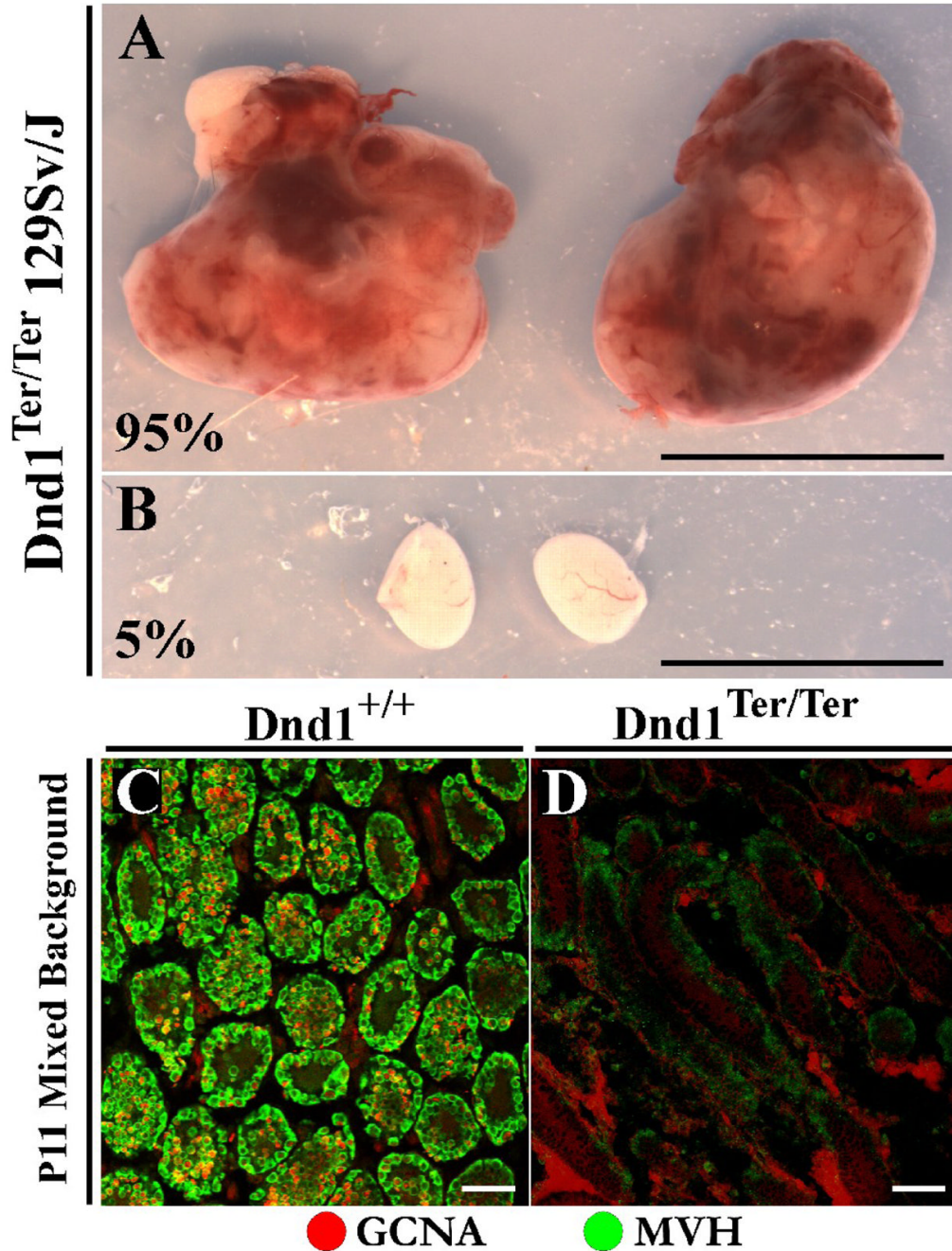


Figure 2. Teratomas form in mutants on the 129Sv/J background but germ cells are lost completely on other genetic backgrounds

(A) Large bilateral testicular teratomas from a P17 129Sv/J *Dnd1^{Ter/Ter}* mutant compared to (B) a mutant from the same litter that did not develop teratomas. On the 129Sv/J background testes develop teratomas at a rate of ~95% while the remainder simply lose their germ cells. Scale bars represent 1cm. (C) P11 wildtype testis section in which germ cells within testis cords are immunostained with antibodies against GCNA (red) and MVH (green). (D) P11 *Dnd1^{Ter/Ter}* testis section on a mixed genetic background contains no germ cells that stain positive for either GCNA or MVH within testis cords by this stage. Scale bars represent 50 μ m.

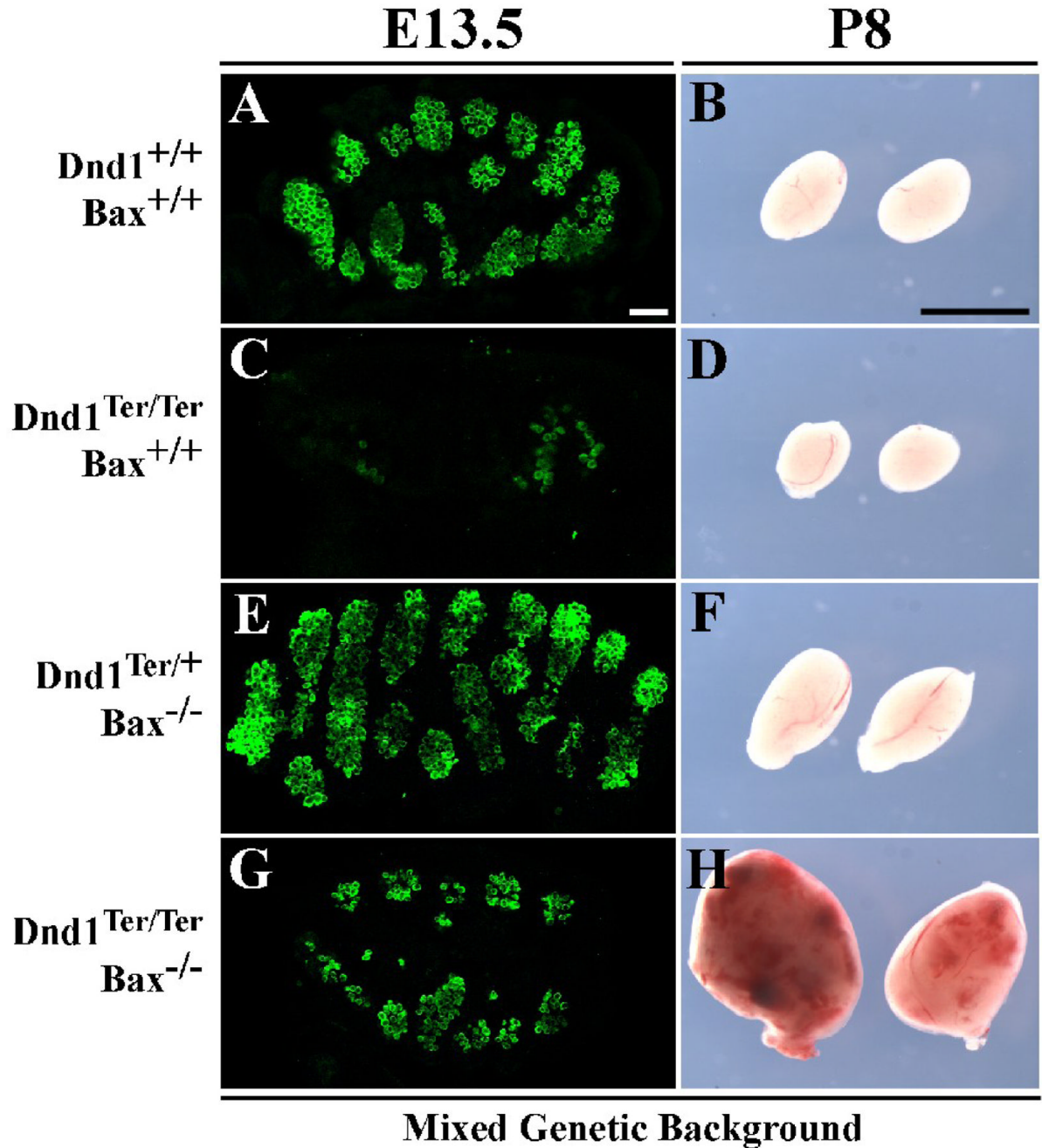


Figure 3. On a *Bax* mutant background, germ cells are partially rescued in *Dnd1*^{Ter/Ter} embryos, and these double-mutants develop testicular teratomas on a mixed genetic background (A) Germ cells (immunostained with MVH in green) are grouped inside testis cords in a wildtype testis at E13.5. Germ cell numbers are greatly reduced in a *Dnd1*^{Ter/Ter} testis (C), and somewhat increased in a *Dnd1*^{Ter/+} mouse carrying two loss of function alleles of *Bax* (E). (G) A *Dnd1*^{Ter/Ter};*Bax*^{-/-} double-mutant testis shows a significant increase in germ cell number compared to C. Scale bars represent 50 μ m. (B) P8 wildtype testes. On a mixed genetic background, *Dnd1*^{Ter/Ter} mutant testes are reduced in size as a result of germ cell loss (D as compared to B). Loss of *Bax* on a *Dnd1*^{+/+} background leads to an increase in testis size (F),

whereas loss of *Bax* on a *Dnd1^{Ter/Ter}* background results in teratoma formation (**H**). Scale bars represent 0.5cm.

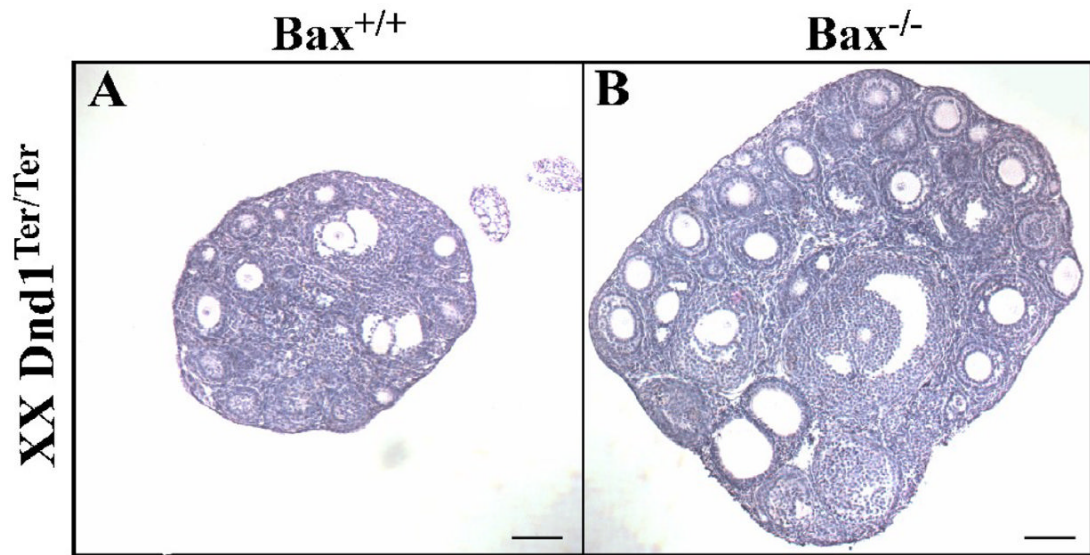


Figure 4. *Dnd1^{Ter/Ter};Bax^{-/-}* ovaries have more oocytes than *Dnd1^{Ter/Ter}* ovaries
 Representative sections from the thickest part of (A) a P16 *Dnd1^{Ter/Ter}* ovary and (B) a P18 *Dnd1^{Ter/Ter};Bax^{-/-}* ovary stained with hematoxylin and eosin. Scale bars represent 100 μm.

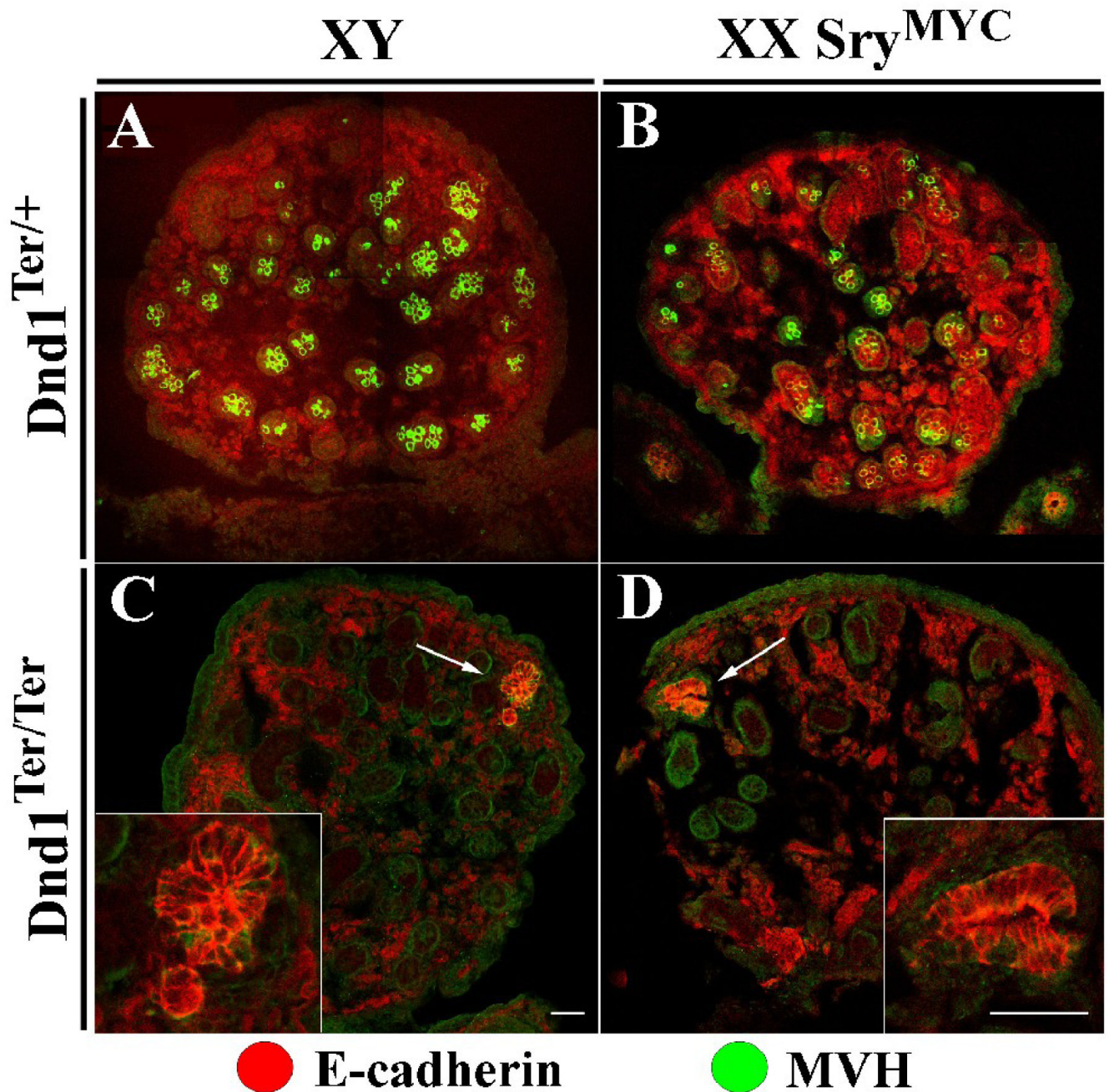


Figure 5. XX germ cells in an XX *Sry^{MYC}* testis form neoplastic clusters like XY germ cells in a testis

XY germ cells are MVH positive (green) in an E18.5 control XY testis (A), and in an XX *Sry^{MYC}* (sex-reversed) testis (B) although their numbers are somewhat reduced. (C) At E18.5, the XY *Dnd1^{Ter/Ter}* testis lacks MVH-positive cells, but an E-cadherin-positive (red) neoplastic cluster is detected. These clusters typically give rise to nascent teratomas (Cook et al., in preparation). (D) At E18.5 the XX *Sry^{MYC} Dnd1^{Ter/Ter}* testis also lacks MVH-positive germ cells but contains a similar E-cadherin positive neoplastic cluster. All testis samples are on a 129Sv/J background. Scale bars represent 50 μ m.

Table 1

Teratoma penetrance in double-mutant gonads on a mixed genetic background.

Genotype	Frequency	Characterization
<i>Dnd1^{Ter/Ter} Bax^{-/-}</i>	91% (20/22)	large, bilateral
<i>Dnd1^{Ter/Ter} Bax^{+/-}</i>	52% (12/23)	small, unilateral
<i>Dnd1^{Ter/Ter} Bax^{+/+}</i>	0% (0/10)	-----

Table 2
Comparison of genotypes from 129Sv/J-*Ter* derived progeny.

	Genotype		Total no. examined	χ^2	<i>P</i>
	+/+	<i>Ter</i> +			
Observed	124	249	500	0.044	0.978
Expected	125	250			