Parent-of-origin effects of A1CF and AGO2 on testicular germ-cell tumors, testicular abnormalities, and fertilization bias

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Testicular tumors, the most common cancer in young men, arise from abnormalities in germ cells during fetal development. Unconventional inheritance for testicular germ cell tumor (TGCT) risk both in humans and mice implicates epigenetic mechanisms. Apolipoprotein B mRNA-editing enzyme complex 1 (APOBEC1) cytidine deaminase and Deadend-1, which are involved in C-to-U RNA editing and microRNA-dependent mRNA silencing, respectively, are potent epigenetic modifiers of TGCT susceptibility in the genetically predisposed 129/Sv inbred mouse strain. Here, we show that partial loss of either APOBEC1 complementation factor (A1CF), the RNA-binding cofactor of APOBEC1 in RNA editing, or Argonaute 2 (AGO2), a key factor in the biogenesis of certain noncoding RNAs, modulates risk for TGCTs and testicular abnormalities in both parent-of-origin and conventional genetic manners. In addition, non-Mendelian inheritance was found among progeny of *A1cf* and *Ago2* mutant intercrosses but not in backcrosses and without fetal loss. Together these findings suggest nonrandom union of gametes rather than meiotic drive or preferential lethality. Finally, this survey also suggested that A1CF contributes to long-term reproductive performance. These results directly implicate the RNA-binding proteins A1CF and AGO2 in the epigenetic control of germ-cell fate, urogenital development, and gamete functions.

A1CF | AGO2 | testicular cancer | parent-of-origin effects | epigenetic inheritance

The germline is the only cell lineage that transmits genetic and epigenetic information across generations. Early in mammalian development, primordial germ cells (PGCs) escape a somatic fate to become unipotent precursors of gametes, the highly specialized cells that give rise to the totipotent zygote upon fertilization (1). Various molecular mechanisms regulate pluripotency by modulating gene expression and protein activity throughout development (2). Failure of pluripotency control can lead to infertility, carcinoma in situ, gamete dysfunctions, and unusual modes of inheritance. Carcinoma in situ anomalously express markers of pluripotency and can give rise to testicular germ cell tumors (TGCTs) (3–7). Studying the genetics, epigenetics, and biology of germ cells (GCs) and TGCTs can provide unique insights about GC development, pluripotency control, tumorigenesis, and unconventional inheritance.

TGCTs are the third most heritable cancer and are the most common cancers in young men 15–35 y old (8). Genome-wide association studies (GWAS) in humans identified susceptibility loci such as KIT ligand (*KITL*), Sprouty 4 (*SPRY4*), Bcl2 antagonist killer (*BAK1*), Doublesex- and Mab3-related transcription factor (*DMRT1*), Deleted in azoospermia RNA-binding protein (*DAZL*), PRDM transcriptional regulator (*PRDM14*), the telomerase reverse transcriptase *TERT*, and its cofactor *AFT7IP* (9–15). Individually and collectively, however, these susceptibility genes account for only a modest portion of inherited risk. Many genes and inherited factors remain to be discovered, their functions in normal development characterized, and the ways that dysfunction leads to TGCTs investigated (16, 17).

Risk for TGCTs is strongly associated with various testicular abnormalities (TAs) such as undescended testis (cryptorchism) and testicular atrophy (18–23). This association, sometimes referred to as "testicular dysgenesis syndrome," suggests shared genetic and environmental origins for TGCTs and abnormalities in urogenital development (24–26).

Studies of human pathologies such as TGCTs occasionally reveal unusual modes of inheritance such as parent-of-origin (PofO) effects, which are implicated when phenotypes are transmitted preferentially through either the maternal or paternal germline (27). Such inheritance is associated with several human conditions (28–30). PofO effects include a four- to sixfold elevated risk of TGCTs among sons of affected versus unaffected fathers (31, 32), inheritance of *SPRY4* risk through the maternal but not paternal germline (15), and gender-specific inheritance of methylation in TGCT families (33). Studying the molecular bases of unconventional inheritance and their associations with pathologies such as TGCTs is challenging in humans because of the need to obtain multigeneration families and to resolve heterogeneity and stratification in study populations. Animal models, with their defined genetics and controlled husbandry, can resolve some of these challenges.

Unlike other inbred strains, males of the 129/Sv family of mouse strains have a strong genetic predisposition to spontaneous TGCTs (Mouse Tumor Biology Database, tumor.informatics.jax.org/mtbwi/index.do) (3, 34). Interestingly, these TGCTs share

Significance

Usually diagnosed in young men, testicular germ cell tumors (TGCTs) originate from abnormalities in germ cells during fetal development. Testicular cancer is a complex disease combining multiple genetic variants and environmental factors. The discovery of unconventional inheritance for TGCT risk both in humans and mice highlighted the major contribution of epigenetic mechanisms. The current work identifies two TGCT modifiers, the RNA-binding proteins apolipoprotein B mRNA-editing enzyme complex 1 (APOBEC1) complementation factor (A1CF) and Argonaute 2 (AGO2), respectively involved in RNA editing and RNA silencing. These results help us better understand the epigenetic control of germ-cell fate, urogenital development, and gamete functions.

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many characteristics with pediatric TGCTs and nonseminomas in humans, including embryonic origin, heterogeneous cell and tissue composition, and abnormal expression of pluripotency markers (7, 35–37). Genetic studies with 129/Sv males have identified many susceptibility genes such as *Kitl*, the RNA-binding protein (RBP) Deadend homolog 1 (*Dnd1*), apolipoprotein B mRNA-editing enzyme complex 1 (*Apobec1*) cytidine deaminase, and the transcriptional factors *Trp53* and *Dmrt1* (3, 38–44). The association between *Kitl* mutations and TGCT susceptibility in mice was later demonstrated in humans, where inherited KITL variants show the strongest association with TGCTs of any GWAS locus (9, 10, 12, 15).

Unusual modes of inheritance such as PofO and transgenerational epigenetic effects are readily characterized with mouse TGCT models (45, 46). For instance, $Sl^{gb/+}$ heterozygous mutant males that carry a *Kitl* deletion transmit strong protection to wild-type male offspring (47). In addition, an engineered loss-of-function *Apobec1* mutation shows contrasting effects on TGCT risk among $Apobec1^{KO/+}$ male offspring depending on whether the $Apobec1^{KO}$ allele is inherited paternally (enhanced risk) or maternally (reduced risk) (41). Maternal $Apobec1^{KO/+}$ heterozygosity also acts in a PofO and transgenerational manner to reduce risk among wild-type male offspring for several generations (41).

Atypical patterns of inheritance can also result from transmission ratio distortion (TRD), which occurs when allelic transmission to offspring departs significantly from Mendelian expectations (48). Examples have been described in mice, flies, and other species, although evidence for strong TRD in humans is weak (49–52). TRD may arise at different stages of male and female gametogenesis (meiotic drive), at fertilization (gamete competition), and during embryonic development (preferential lethality). Mechanisms underlying such events may be allele-, sex-, or strain-specific (50, 52). In mice, TRD also has been reported in intercrosses with mutant heterozygotes for TGCT-susceptibility genes such as $Dnd1^{tm1Na}$ (hereafter referred to as "$Dnd1^{KO}$") (53) and a combination of maternal $Apobec1^{KO/+}$ and paternal $Dnd1^{Ter/+}$ heterozygosity (41). Together, these results suggest that susceptibility genes for TGCTs may also affect gamete functions in ways that bias genetic transmission.

Jablonka and Lamb (54) proposed that anomalies in the epigenetic regulation of the germline could lead to TRD and infertility. The present study tested the role of two epigenetic factors, namely APOBEC1 complementation factor (A1CF), the RNA-binding cofactor for APOBEC1 in C-to-U RNA editing (55, 56), and Argonaute 2 (AGO2, also known as "EIF2C2"), a key factor of microRNA (miRNA)- and siRNA-mediated gene silencing, on TGCT susceptibility. The DND1 protein shares sequence similarity with A1CF (40). Consequently, DND1 could affect TGCT susceptibility through effects on mRNA editing. Indeed, APOBEC1 is a potent TGCT modifier of parental effects, gametic transmission, and transgenerational epigenetic inheritance (41). Interestingly, $A1cf^{KO/+}$ heterozygous matings also show strong TRD (57). A1CF and APOBEC1 therefore may have similar effects on TGCTs and TRDs. Here we tested the consequences of partial A1CF deficiency on the susceptibility to TGCTs, TAs, and TRDs as well as on epigenetic inheritance in genetically predisposed 129/Sv mice.

In parallel, previous work showed that DND1 directly binds the 3′ UTR of specific mRNAs, thereby blocking access of miRNAs to their targets in TGCT cell lines (58). DND1 associates with several pluripotency transcripts such as OCT4, NANOG, and lineage defect LIN28 (59). If DND1 contributes to TGCT susceptibility by interfering with miRNA functions, genes directly involved in miRNA biogenesis should have similar effects. AGO2 regulates miRNA and endogenous siRNA functions (60). To determine whether miRNA and siRNA pathways are directly implicated in teratocarcinogenesis and related aspects of GC biology, we tested the effects of partial AGO2 deficiency on susceptibility to TGCTs, TAs, and TRDs and on PofO effects in 129/Sv mice.

We found that both *A1cf* and *Ago2* reduce the risk for TGCTs in both PofO and conventional manners, regulate TA susceptibility, and show TRD, albeit in somewhat different manners. Together, these results support the role of epigenetics on mRNA availability for translation as well as the link between unconventional inheritance and biased fertilization.

Results

Study Design. The purpose of this survey was to test the impact of *A1cf* and *Ago2* hemizygosity on parental versus conventional inheritance of TGCT susceptibility, transmission ratios, and reproductive performance over three backcross generations. For both mutants, a combination of reciprocal backcrosses and intercrosses was used to assess inheritance of TGCT and TA risk. $A1cf^{KO/+}$ and $Ago2^{KO/+}$ mutant mice were generated from related 129-derived targeted ES cell lines (57, 61) and then were backcrossed to inbred 129/Sv control mice. A total of 1,589 offspring males, including 361 from 129/Sv control crosses, 1,010 from separate $A1cf^{KO/+}$ and $Ago2^{KO/+}$ reciprocal backcrosses, and 218 males from separate $A1cf^{KO/+}$ and $Ago2^{KO/+}$ intercrosses, were examined for TGCTs and TAs (Tables 1 and 2 and Table S1). Conventional (Mendelian) inheritance was inferred in cases in which maternal and paternal inheritance affected offspring phenotypes similarly. By contrast, a PofO effect was inferred in cases in which offspring phenotype depended on parental sex and genotype.

PofO Effects on TGCT Susceptibility. Growing evidence of PofO effects on TGCT risk in both humans (15, 31, 32) and mice (41, 47, 63) suggests that epigenetic mechanisms influence tumorigenesis. Here, we asked whether *A1cf* and *Ago2* contribute to TGCT susceptibility in a conventional or a PofO manner.

129/Sv. Because the occurrence of TGCT-affected males in the present survey (6.9%) (Table 1) was consistent with previous reports (34, 41, 62, 63), the 7% long-term average was used to analyze *A1cf* and *Ago2* results.

A1cf. Partial deficiency for *A1cf* had both conventional and PofO effects, depending on parental and offspring genotype (Table 1). Maternal but not paternal heterozygosity significantly reduced risk in wild-type male offspring (8.6-fold, $P < 0.007$). By contrast, conventional effects were found in $A1cf^{KO/+}$ heterozygous offspring with both maternal and paternal heterozygosity, in which risk was strongly reduced (2.4- and 3.1-fold, $P < 0.06$ and <0.03, respectively). TGCT occurrence among $A1cf^{KO/+}$ intercross progeny did not differ significantly from the 129/Sv baseline.

Ago2. A protective PofO effect was found in $Ago2^{KO/+}$ backcrosses in which the occurrence of affected $Ago2^{KO/+}$ heterozygous males was reduced significantly with maternal but not paternal heterozygosity (6.3-fold, $P < 0.03$) (Table 1). No other backcross or intercross results differed significantly from 129/Sv.

Thus, both *A1cf* and *Ago2* affected TGCT risk, depending on parental sex and offspring genotype. As with $Apobec1^{KO/+}$ heterozygosity (41), maternal$A1cf^{KO/+}$ heterozygosity reduced risk among all male offspring, whereas paternal heterozygosity led to conventional genetic effects, albeit in a different direction (reduced risk) than results for *Apobec1* (increased risk).

PofO Effects on TA Susceptibility. In humans, cryptorchism is relatively common (3–5%); atrophy and agonadism are less common (0.2%) (64). We investigated whether partial deficiency of *A1cf* or *Ago2* affected TA incidence in the same crosses in which the TGCT survey was conducted.

129/Sv. The occurrence of cryptorchid as well as atrophic testes (including agonadism) among 129/Sv males (18.3%) (Table S1) was consistent with the 18% rate previously reported (65).

A1cf. A strong paternal effect was found in $A1cf^{KO/+}$ backcrosses in which heterozygous male offspring showed a 3.7-fold reduced risk for atrophy ($P < 0.006$) (Table 2). By contrast, a conventional effect for atrophy was observed among wild-type offspring with

Table 1. Occurrence of TGCT-affected males in the 129/Sv control strain and in *A1cf*$^{KO/+}$ and *Ago2*$^{KO/+}$ reciprocal backcrosses and intercrosses

Crosses	Offspring genotype	No. males	Affected males		Test score χ^2, *P*-value	Conclusion (fold-change)
			No.	Frequency, %		
129/Sv control strain						
129/Sv × 129/Sv	Wild-type	361	25	6.9	<0.1, NS	Comparable to the published rate
A1cf$^{KO/+}$ test crosses						
A1cf$^{KO/+}$ × 129/Sv	**Wild-type**	**126**	**1**	**0.8**	**7.4, <0.007**	**Maternal heterozygosity reduced risk in all progeny (−8.6-fold for wild-type; −2.4-fold for heterozygotes)**
	***A1cf*$^{KO/+}$**	**138**	**4**	**2.9**	**3.8, <0.06**	
129/Sv × *A1cf*$^{KO/+}$	Wild-type	107	5	4.7	1.1, NS	No paternal effect
	***A1cf*$^{KO/+}$**	**136**	**3**	**2.2**	**5.0, <0.03**	**Paternal heterozygosity reduced risk in *A1cf*$^{KO/+}$ progeny (−3.1-fold)**
A1cf$^{KO/+}$ × *A1cf*$^{KO/+}$	Wild-type	41	1	2.4	0.8^{y}, NS	No effect on susceptibility in intercross progeny
	A1cf$^{KO/+}$	107	5	4.7	1.1, NS	
Ago2$^{KO/+}$ test crosses						
Ago2$^{KO/+}$ × 129/Sv	Wild-type	115	5	4.3	1.5, NS	No maternal effect
	***Ago2*$^{KO/+}$**	**91**	**1**	**1.1**	**4.9, <0.03**	**Maternal heterozygosity reduced risk in *Ago2*$^{KO/+}$ progeny (−6.3-fold)**
129/Sv × *Ago2*$^{KO/+}$	Wild-type	144	6	4.2	2.1, NS	No paternal effect
	Ago2$^{KO/+}$	153	5	3.3	3.6, NS	
Ago2$^{KO/+}$ × *Ago2*$^{KO/+}$	Wild-type	37	2	5.4	<0.1^{y}, NS	No effect on susceptibility in intercross progeny
	Ago2$^{KO/+}$	33	2	6.1	<0.1^{y}, NS	

A total of 1,589 offspring males, including 361 from 129/Sv control crosses, 1,010 from separate *A1cf*$^{KO/+}$ and *Ago2*$^{KO/+}$ reciprocal backcrosses, and 218 males from separate *A1cf*$^{KO/+}$ and *Ago2*$^{KO/+}$ intercrosses, were examined for TGCTs and TAs (Tables 1 and 2 and Table S1). Conventional (Mendelian) inheritance was inferred in cases where maternal and paternal inheritance affected offspring phenotypes similarly. By contrast, a PofO effect was inferred in cases where offspring phenotype depended on parental sex and genotype. χ^2 goodness-of-fit tests were used to compare the occurrence of TGCT-affected heterozygous and wild-type males with the 7% baseline in the 129/Sv inbred strain (34, 41, 62, 63). χ^2 (χ^2) and *P* values are indicated for each test result (df = 1). Results below the pointwise 0.05 threshold are highlighted in bold font with a gray background. We treated the six tests for each mutant (*A1cf* and *Ago2*) as a "family" of tests. Results that showed family-wide significance at an FDR of 0.1 are underlined. Fold-change refers to results for each mutant test cross versus the 129/Sv control strain. Results highlighted in bold (no gray, no underlining) represent a strong trend with substantial fold-change. NS indicates results that did not pass the threshold of statistical significance. y indicates Yate's correction was applied to the test.

occurrence strongly reduced (2.3- and 3.9-fold, $P < 0.05$ and $P < 0.02$, respectively). TA occurrence among *A1cf*$^{KO/+}$ intercross progeny did not differ significantly from the 129/Sv rate (Table 2). Cryptorchism occurred at comparable frequencies in all *A1cf*$^{KO/+}$ crosses and 129/Sv (Table S1).

Ago2. Partial deficiency of *Ago2* did not significantly affect the occurrence of cryptorchism or atrophy in *Ago2*$^{KO/+}$ backcross progeny (Table S1). However, wild-type and heterozygous intercross progeny showed a 2.6- and 2.5-fold increased risk for cryptorchid testes compared with 129/Sv mice (Table 2).

Table 2. Effect of *A1cf*^{KO} and *Ago2*^{KO} on occurrence of atrophy and cryptorchism in backcross and intercross progeny

Crosses	Offspring genotype	No. males	Affected males		Test score χ^2, *P*-value	Conclusion (fold-change)
			No.	Frequency, %		
Atrophy						
129/Sv × 129/Sv	Wild-type	361	39	10.8		
A1cf$^{KO/+}$ × 129/Sv	**Wild-type**	**126**	**6**	**4.8**	**4.1, <0.05**	**Maternal heterozygosity reduced risk in wild-type progeny (−2.3-fold)**
	A1cf$^{KO/+}$	138	9	6.5	2.1, NS	
129/Sv × *A1cf*$^{KO/+}$	**Wild-type**	**107**	**3**	**2.8**	**6.5, <0.02**	**Paternal heterozygosity reduced risk in all progeny (−3.9- and −3.7-fold)**
	***A1cf*$^{KO/+}$**	**136**	**4**	**2.9**	**7.7, <0.006**	
A1cf$^{KO/+}$ × *A1cf*$^{KO/+}$	Wild-type	41	3	7.3	0.2^{y}, NS	No effect on susceptibility in intercross progeny
	A1cf$^{KO/+}$	107	6	5.6	2.6, NS	
Cryptorchism						
129/Sv × 129/Sv	Wild-type	361	26	7.2		
Ago2$^{KO/+}$ × 129/Sv	Wild-type	115	12	10.4	1.2, NS	No maternal effect
	Ago2$^{KO/+}$	91	4	4.4	0.9, NS	
129/Sv × *Ago2*$^{KO/+}$	Wild-type	144	10	6.9	<0.1, NS	No paternal effect
	Ago2$^{KO/+}$	153	12	7.8	<0.1, NS	
Ago2$^{KO/+}$ × *Ago2*$^{KO/+}$	**Wild-type**	**37**	**7**	**18.9**	**4.6^{y}, <0.04**	**Increased risk in all progeny (+2.6- and +2.5-fold)**
	***Ago2*$^{KO/+}$**	**33**	**6**	**18.2**	**3.5^{y}, NS**	

Comparison of results for *A1cf*$^{KO/+}$, *Ago2*$^{KO/+}$ and respective wild-type sibling males in reciprocal backcrosses and intercrosses with results for males in the 129/Sv control strain. χ^2 contingency test with an FDR assessment. Bold, underlining, and shading are as in Table 1. See Table 1 for additional information. Complete data are supplied in Table S1. NS, not significant.

Thus, both *A1cf* and *Ago2* affected the occurrence of TAs in 129/Sv mice but did so in contrasting ways: *A1cf* significantly reduced the risk of atrophy in backcross progeny in both conventional and PofO manners, whereas $Ago2^{KO/+}$ heterozygosity increased the risk of cryptorchism in wild-type and heterozygous mutant intercross progeny.

Cooccurrence of TGCTs and TAs. In humans, individuals with a cryptorchid testis have an elevated risk of developing additional urogenital conditions including reduced fertility, testicular atrophy, and TGCTs (18, 21). An estimated 10% of testicular tumors are associated with cryptorchid testis (18). In all crosses, we found males with cryptorchid or atrophic testes that also had a TGCT, referred to hereafter as "cryptorchid TGCT" and "atrophic TGCT" cases, respectively. However, because such cases were rare in $A1cf^{KO/+}$ and $Ago2^{KO/+}$ crosses, we restricted the analysis to the 129/Sv strain (Table S2).

Interestingly, we found the joint occurrence of cryptorchism and TGCTs was increased 3.9-fold over the expectations for independent risks ($P < 0.0002$) (Table S2). In fact, 27% of cryptorchid cases also had a TGCT. The association between cryptorchism and TGCT in the same testis is consistent with observations in humans (19, 20). By contrast, atrophy and TGCTs occurred independently (Table S2). Also, TGCTs with contralateral atrophy were rare, and cryptorchism was never associated with contralateral TGCTs; this finding is consistent with the low risk for TGCTs in scrotal testes with a contralateral cryptorchid testis in humans (66).

Risk for Male Offspring of Affected Males. In humans, sons of a father with a TGCT or a cryptorchid testis have an elevated risk for TGCTs and for cryptorchism (four- to sixfold and four- to fivefold, respectively) (31, 32, 67). In our study, the presence of paternal TAs was not associated with altered risk for TGCTs or TAs among progeny (see Table S4). Therefore, we pooled the results for sons of male parents with healthy testes together with those of sons of male parents affected with a TA. This pool, hereafter referred to as "healthy breeders," was then used to test the effect of filial relations on TGCT risk.

Although results are anecdotal because of the modest number of breeders with TGCT-affected parental males (referred to hereafter as "TGCT breeders") (Table S3), the similarity with evidence from humans is striking. The first example involves progeny of an affected $A1cf^{KO/+}$ breeder male: 21.4% of $A1cf^{KO/+}$ male offspring developed a TGCT, whereas only 2.2% of the progeny of healthy breeders were affected—a 9.7-fold difference ($P < 0.006$) (Table S3). The second example involves progeny of a TGCT-affected 129/Sv control male mated with $Ago2^{KO/+}$ females. TGCT risk among $Ago2^{KO/+}$ male offspring of this cross increased 15.2-fold compared with progeny of healthy breeders ($P < 0.04$) (Table S3). If validated in a larger study, these results suggest that the action of a paternally inherited factor depends on offspring genotype, because in both examples, increased risk was found in mutant heterozygous offspring but not in their wild-type siblings (Table S3).

No significant differences in TA occurrence were detected between the 118 male offspring of TGCT breeders and the progeny of healthy breeders (Table S4).

Laterality. Human TGCTs are generally unilateral (21), with no obvious side preference (68). By contrast, mouse TGCTs present a 2:1 left:right bias (3, 34, 65) that is accentuated in some strains such as $Dnd1^{Ter}$ (34, 69, 70). However, *A1cf* and *Ago2* partial deficiencies did not affect laterality (Table S5). Bilateral cases were rare (3, 21, 65, 70). Cryptorchism and atrophy occurred predominantly on the left side without a significant difference between 129/Sv and the other strains, also confirming previous reports (3, 34, 65). Bilateral TAs were infrequent (Table S5), whereas in humans 15% of all cryptorchid testes are bilateral (64). Thus, both TGCTs and TAs in mice are primarily unilateral with a strong left-preference that is largely unaffected by partial loss of *A1cf* or *Ago2* function.

TRD. Transmission of alternative alleles from heterozygotes is usually Mendelian, but exceptions are known at selected loci in several species (49–51). For example, a fivefold excess of heterozygotes over expectations is found in $A1cf^{KO/+}$ intercrosses (57) and was confirmed in the current survey ($P < 0.002$) (Table 3). We also observed TRD in $Ago2^{KO/+}$ crosses, with only 50% of the expected number of $Ago2^{KO/+}$ heterozygotes among progeny of both intercrosses ($P < 0.0002$) (Table 3 and Table S6) and backcrosses with maternal but not paternal $Ago2^{KO}$ heterozygosity ($P < 0.02$) (Table 3). Interestingly, the genotypic bias was stronger for females in $A1cf^{KO/+}$ intercrosses ($P < 0.003$) and for males in $Ago2^{KO/+}$ intercrosses ($P < 0.0006$) (Table S6), suggesting that sex chromosomes may be involved. In all cases, litter sizes were similar among backcross and intercross matings, suggesting that embryonic lethality was not responsible for distorting transmission.

Table 3. $A1cf^{KO/+}$ and $Ago2^{KO/+}$ transmission in backcrosses and intercrosses

Crosses	Litter size ± SEM (no. of litters)	% males	No. (expected) +/+	No. (expected) KO/+	Test score χ^2, *P*-value	Conclusion (fold-change)
129/Sv control strain						
129/Sv × 129/Sv	4.9 ± 1.9 (59)	50.6				
$A1cf^{KO/+}$ test crosses						
$A1cf^{KO/+}$ × 129/Sv	5.7 ± 1.9 (92)	51.1	255 (262.5)	270 (262.5)	0.4, NS	Mendelian
129/Sv × $A1cf^{KO/+}$	5.8 ± 1.8 (86)	50.6	234 (249.5)	265 (249.5)	1.9, NS	Mendelian
$A1cf^{KO/+}$ × $A1cf^{KO/+}$	**5.9 ± 1.6 (51)**	**48.7**	**75 (101.3)**	**229 (202.7)**	**10.2, <0.002**	**Excess of heterozygotes (+1.5 fold)**
$Ago2^{KO/+}$ test crosses						
$Ago2^{KO/+}$ × 129/Sv	**4.8 ± 1.8 (90)**	**47.9**	**241 (216)**	**191 (216)**	**5.7, <0.02**	**Deficiency of heterozygotes (−1.3 fold)**
129/Sv × $Ago2^{KO/+}$	4.9 ± 1.8 (126)	49.3	314 (310.5)	307 (310.5)	<0.1, NS	Mendelian
$Ago2^{KO/+}$ × $Ago2^{KO/+}$	**3.9 ± 1.7 (33)**	**53.8**	**64 (43.3)**	**66 (86.7)**	**14.8, <0.0002**	**Deficiency of heterozygotes (−1.9 fold)**

Genotypic transmission in progeny compared with Mendelian expectations (1:1) for $A1cf^{KO/+}$ and $Ago2^{KO/+}$ backcrosses and (1:2) for intercrosses. Sexes are similarly represented (not shown); only combined data are presented. χ^2 goodness-of-fit test. Bold, underlining, and shading are as in Table 1. Complete data are given in Table S6.

Table 4. Reproductive performance in 129/Sv control cross and *A1cf*$^{KO/+}$ and *Ago2*$^{KO/+}$ backcrosses

Crosses	No. of breeders	Mean interval	Average litter size			Litter index	*P*-value	Conclusion
			First	Last	*P*-value			
129/Sv control strain								
129/Sv × 129/Sv	17	21.9 ± 1	5.4 ± 1	4.3 ± 2		0.59		
A1cf$^{KO/+}$ test crosses								
A1cf$^{KO/+}$ × 129/Sv	11	23.7 ± 2	5.9 ± 2	6.1 ± 2	<0.04	0.68	NS	Maternal heterozygosity improves reproductive performance
129/Sv × *A1cf*$^{KO/+}$	**10**	**24.5 ± 4**	**6.0 ± 2**	**6.7 ± 1**	**<0.0006**	**0.71**	**<0.04**	**Paternal heterozygosity improves reproductive performance**
Ago2$^{KO/+}$ test crosses								
Ago2$^{KO/+}$ × 129/Sv	14	22.7 ± 1	5.9 ± 2	4.8 ± 1	NS	**0.50**	**<0.05**	No maternal effect
129/Sv × *Ago2*$^{KO/+}$	16	22.3 ± 2	5.8 ± 1	5.7 ± 2	NS	0.57	NS	No paternal effect

Mean interval (days) between mating and first litter, average size of first and last litters, and litter index are compared in 129/Sv control cross and *A1cf*$^{KO/+}$ and *Ago2*$^{KO/+}$ backcrosses. *t* tests. Only significant results are presented. Bold, shading, and underlining are as in Table 1.

Gametogenesis and Reproduction. TRD may arise during gametogenesis, at fertilization, or during embryogenesis. We therefore examined morphological and histological features of oogenesis, spermatogenesis, and reproductive performance of *A1cf* and *Ago2* mutant mice.

Oogenesis. Given that puberty (and therefore first ovulation) occurs at ∼29 d of age (71), the total number of eggs present at birth and fixed for the lifetime can be reliably assessed in females at weaning. On average in prepubertal *A1cf*$^{KO/+}$, *Ago2*$^{KO/+}$, and 129/Sv ovaries, we counted 45–47 eggs/mm^2 (Fig. S1*A*). However, two (of 15) *A1cf*$^{KO/+}$ ovaries had dramatically more eggs (141 eggs/mm^2; $P \sim 0$), suggesting heterogeneity in reproductive performance among *A1cf*$^{KO/+}$ females, although no outlier litter sizes were noted. Oocyte maturation was assessed with emphasis on primary, secondary, early antral, and antral follicles, but no significant differences were observed (Fig. S1*A*). The number of corpora lutea in adult ovaries did not vary substantially, suggesting quantitatively normal ovulation in the three strains. Overall histology also appeared normal (Fig. S1*A*).

Spermatogenesis. Testis weight is an established proxy measure of spermatogenesis and male fertility (72, 73). The average body weight of adult *A1cf*$^{KO/+}$ and *Ago2*$^{KO/+}$ males (23.2 and 22.5 g, respectively) did not differ significantly from that of 129/Sv controls (23.7 g) (Fig. S1*B*). 129/Sv males showed a gonad/body mass (G/B) ratio of 4.3 that was not significantly affected by partial deficiency of *A1cf* or *Ago2* (4.3 and 4.4, respectively). Histological analysis of *A1cf*$^{KO/+}$ and *Ago2*$^{KO/+}$ testes revealed no obvious abnormalities. Mature spermatozoa in the three strains had normal morphology (Fig. S1*B*), although *A1cf*$^{KO/+}$ adult males produced fewer mature sperm (276×10^6 sperm/mL) than 129/Sv and *Ago2*$^{KO/+}$ adult males (414 and 345×10^6 sperm/mL, respectively) (Fig. S1*B*).

Reproduction. The number of pups per litter at weaning, the age at first litter, litter intervals, and persistence of productivity are commonly used to characterize reproductive performance (74). Mating and culling times were set up blinded to genotype. No significant differences in parental age at mating, death, or at first and last litters were observed among strains. The litter interval between mating and the first litter did not differ significantly among strains (Table 4). Most breeders appeared to be still productive at the time they were killed, making an estimate of the reproductive lifespan for each strain impossible. Nonetheless, the reproductive capacity of 129/Sv controls was declining at the time of death, with one less pup in the last litter than in the first litter (Table 4), as expected in aging laboratory mice (75) and suggesting that the end of breeding productivity for our strains was imminent.

Interestingly, although the size of first litters did not differ significantly among strains (Table 4), the number of pups in the last litters was significantly higher in *A1cf* backcrosses: +1.8 ($P < 0.04$) and +2.4 pups ($P < 0.0006$) with maternal and paternal heterozygosity, respectively (Table 4), compared with 129/Sv control cross and *Ago2* backcrosses (Table 4). Consequently, average litter sizes were increased by approximately one pup in all *A1cf* crosses (Table 3) compared with 129/Sv control cross and *Ago2* backcrosses and despite the early lethality of *A1cf*$^{KO/KO}$ homozygotes in intercross (57). By contrast, *Ago2* intercrosses lost one pup on average per litter (Table 3), in accordance with the early embryonic lethality of *Ago2*$^{KO/KO}$ homozygotes (61).

Furthermore, litter indexes were similar in 129/Sv control crosses (0.59) (Table 4) and *Ago2* backcrosses (0.50 and 0.57 with maternal and paternal heterozygosity, respectively) (Table 4) but were markedly increased in *A1cf* backcrosses (0.68 and 0.71 with maternal and paternal heterozygosity, respectively) (Table 4).

Thus, no obvious quantitative or histological evidence for the effects of partial deficiency of *A1cf* and *Ago2* on oogenesis and spermatogenesis was found in 129/Sv mice. Heterozygous males and females were fully fertile with seemingly normal gonads and GCs, despite a reduced number of *A1cf*$^{KO/+}$ adult sperm. Surprisingly, however, *A1cf*$^{KO/+}$, but not *Ago2*$^{KO/+}$, heterozygosity improved the reproductive performance of the aging 129/Sv inbred strain.

Expression of *A1cf* in Developing and Mature 129/Sv Gonads. A1CF is highly expressed in the kidney, liver, and small intestine of adult mice and humans and also in heart, spinal cord, and lung of mouse embryos at embryonic day 12.5 (E12.5) (57, 76, 77). A1CF transcripts also were detected in gonads of adult humans (77). Here, the presence of *A1cf* transcripts was confirmed in 129/Sv muscle and liver (76) at birth [postnatal day 0 (P0)], P21, and P71 (Fig. S2). *A1cf* transcripts also were detected in 129/Sv testes, epididymides, and ovaries at all time points (Fig. S2).

A1CF protein expression then was investigated in the same tissues and at the same time points (Fig. S3). Staining in the liver was strong in both cytoplasm and nucleus at P0 and P21 but was largely circumscribed within nucleus of hepatocytes in adults, in accordance with previous studies (76, 78). Similarly, weak expression of A1CF in muscle (76) was confirmed at all time points in our study (Fig. S3). Therefore, liver and muscle served as references to assess A1CF expression in test samples (epididymides, testes, and ovaries). In epididymides and testes from 129/Sv mice, A1CF staining was strong in the cytoplasm but was weak in the nuclei of somatic cells at P0 and P21. In adult males, expression was reduced globally with a major cytoplasmic localization in spermatozoa and surrounding somatic cells of both tissues. Finally, A1CF staining was strong in both cellular compartments of oocytes in newborn pups and was weak in surrounding somatic cells. Staining then became saturated in eggs at all stages of maturation in weaning and adult 129/Sv females and

remained relatively strong in surrounding somatic cells within the follicles of P21 and P70 ovaries.

By contrast, AGO2 is widely and ubiquitously expressed in mouse embryos and adults (79–81), as it is in adult humans (Human Protein Atlas, www.proteinatlas.org/). As expected, strong expression of *Ago2* was found in all tissues tested from birth to adulthood (Fig. S2).

In summary, A1CF is strongly expressed at birth in 129/Sv germ and somatic cells of the testes and declines with age. By contrast, A1CF expression increased in maturing eggs from birth to adulthood. These results suggest that A1CF, and especially maternal A1CF, may play a role in gametogenesis, fertilization, and early embryogenesis, either directly or through their downstream actions as RBPs.

Discussion

Inherited genetic and epigenetic information controls fundamental biological processes and phenotypic variation across generations. Genetic and epigenetic anomalies in the germline can lead to testicular cancer, infertility, and unusual modes of inheritance (3–6, 15, 21, 41, 54). The discovery that *Apobec1*, *Dnd1*, and *Eif2s2* are potent modifiers of TGCT susceptibility with both conventional and transgenerational effects highlights the emerging role of RNA editing, miRNA regulation, and RNA availability on GC transformation and epigenetic inheritance (38–41, 63, 82). To explore this issue more deeply, we tested two hypotheses about the role of RNA biology in control of the GC lineage. If RNA editing is indeed involved, as results for APOBEC1 suggest (41), then the A1CF RBP that guides APOBEC1 to specific mRNAs for editing should show similar effects on TGCT risk and epigenetic inheritance. Similarly, if miRNA regulation is critical, as DND1 results suggest (38–40, 63), then AGO2, which regulates mRNA stability based on miRNA and siRNA targeting, should also affect TGCT risk in both conventional and epigenetic manners. As phenotypic outcomes, we focused on TGCT risk, TA abnormalities, and TRDs.

TGCT Risk.

A1cf. A1CF is the RNA-binding cofactor for the APOBEC1 cytidine deaminase in RNA editing (55, 56, 83) and shares sequence similarity with DND1 and several other RBPs (40, 84, 85). We found that, like APOBEC1 (41) and DND1 (40), A1CF regulates TGCT susceptibility with both conventional and PofO effects, consistent with a role for RNA editing in teratocarcinogenesis.

Partial deficiency of *A1cf* and *Apobec1* has similar PofO effects on TGCT susceptibility. Maternal heterozygosity for either of these genes reduced risk among all male offspring, regardless of their genotype (Table 1). By contrast, paternal heterozygosity had disparate consequences on TGCT risk (Table 1), i.e., an increased risk for *Apobec1* and a reduced risk for *A1cf* (Table 1 and ref. 41), suggesting that A1CF and APOBEC1 have distinct context-dependent functions. This hypothesis is supported by the full viability and fertility of APOBEC1-deficient mice (86, 87), whereas A1CF deficiency leads to early embryonic lethality (57).

Furthermore, A1CF was found at varying levels in nucleus and cytoplasm of GCs from birth and throughout adulthood (Fig. S3). Its presence in nuclei as well as cytoplasm suggests that A1CF, like many other RBPs, has multiple functions (84, 85). Given its sequence homology with DND1 (40), A1CF, like DND1, may transport RNAs from nucleus to cytoplasm, in particular to perinuclear P-bodies under stress conditions, and may control access of specific miRNAs to their mRNA targets and perhaps contribute to other aspects of translation arrest (58, 88).

Ago2. AGO2 is an RBP essential for oogenesis (89, 90) and early embryogenesis (61, 91) but is dispensable for spermatogenesis in mice (92). Our study revealed an additional function for AGO2 on GC fate with a strong PofO effect on TGCTs. Indeed, maternal but not paternal $Ago2^{KO}$ heterozygosity reduced risk among heterozygous male offspring (Table 1). This maternal effect may result from monoallelic expression because *Ago2* has characteristics of imprinted genes with a CpG island located within its promotor (−554 to −47 bp from ATG, per CpG islands prediction) that contributes to maternal inheritance in mouse brain and intestinal stem cells (93, 94). However, such monoallelic expression remains to be demonstrated in the mouse germline.

AGO2 is a key factor for siRNA- and miRNA-mediated silencing events that control many downstream pathways (60). miRNA deregulation is an important contributor to tumorigenesis and tumor progression (17, 95). Altered levels of the lethal defect Let-7 miRNA family and LIN28, both regulating pluripotency in growing oocytes and early embryos (96), are characteristics of GC tumors (seminomas and nonseminomas) in humans (17, 95). Interestingly, the TGCT modifier DND1 has been reported to regulate LIN28 transcription (59) that in turn directly controls the expression of the Let-7 family (97–99), supporting the link between miRNAs, pluripotency, and TGCT risk.

Furthermore, siRNAs regulate the expression of transposable elements (TEs) after fertilization and later in primordial GCs (PGCs) (100–102). TEs are heritable mobile genetic elements that can contribute to diseases such as cancer (100, 103). Indeed, altered methylation levels of TEs are commonly found in human tumors such as TGCTs (seminomas and nonseminomas) (33, 104–106). TE regulation is also under the control of RNA editors (ADARs, APOBECs) such as the potent TGCT modifier APOBEC1 (107), emphasizing the role of TEs in TGCT risk and suggesting a functional link between AGO2 siRNAs and APOBEC1-A1CF in teratocarcinogenesis.

Filial relationships. In humans, offspring risk is significantly elevated if the father is affected with a TGCT (seminoma or nonseminoma) (31, 32), but the genetic, epigenetic, and environmental basis for this unusual relationship is uncertain and difficult to investigate in humans. Surprisingly, in our mouse survey we found two examples in maternal $Ago2^{KO/+}$ and paternal $A1cf^{KO/+}$ backcrosses (Table S3). Among offspring of affected males, susceptibility was increased in $Ago2^{KO/+}$ and $A1cf^{KO/+}$ heterozygous offspring but not in their wild-type siblings. These results suggest that factors in the affected paternal germline potentiate the effects of TGCT modifiers such as maternal $Ago2^{KO}$ and paternal $A1cf^{KO}$ when inherited in the subsequent generation.

These paternal factors may act epigenetically to control DNA methylation. Aberrant DNA methylation is associated with familial TGCT susceptibility in humans (33, 108). DNA methyltransferases (DNMTs) are known to be essential for the progression and aggressiveness of tumors such as TGCTs (109–111). In our filial TGCT cases, paternal TGCTs might express factors that indirectly alter the methylation pattern at the promotors of TEs, miRNAs, and siRNAs (33, 112, 113), inherited elements that are direct targets of TGCT modifiers such as AGO2, DND1, and APOBEC1 (58, 60, 107). The modifiers might interpret these inherited epigenetic factors in heterozygous offspring of affected parent males, resulting in an increased susceptibility compared with the wild-type siblings or heterozygous offspring of healthy male parents. Association of TE methylation status with the father–son relationship and TGCT risk in humans supports this hypothesis (33). $A1cf^{KO/+}$ and $Ago2^{KO/+}$ mouse models could help characterize the molecular aspects of familial TGCT cases.

Together, these results show that A1CF and AGO2 are two potent TGCT modifiers, suggesting a crucial role of RNA editing and RNA silencing as well as for miRNAs, siRNAs, and TEs in tumor formation and risk inheritance. More importantly, our study suggests that maternal factors (i.e., the maternal effect of AGO2) strongly contribute to TGCT susceptibility in the subsequent generations and that the TGCT fate of GCs may already be settled in mature eggs. At the same time, factors in affected

male parents contribute to increased risk among genetically predisposed offspring.

TA Risk, Reproductive Performance, and TRD. GCs and surrounding somatic cells (Leydig and Sertoli cells) interact from the earliest stages of the development in the urogenital ridge, ensuring normal development of both cell types (114, 115). For instance, Leydig cells control testis descent and indirectly control spermatogenesis (through interaction with Sertoli cells) (114, 115). Sertoli cells support GC migration, proliferation, and differentiation. Absence of proper cell–cell interactions leads to various gonadal abnormalities such as TGCTs, cryptorchism, and atrophy, suggesting a common developmental etiology (23–25, 114, 115). Although several signaling pathways have been characterized (115), the genetic, epigenetic, and molecular origins of such developmental abnormalities remain unclear. Our results offer insights with the identification of two factors, A1CF and AGO2, that epigenetically modulate phenotypes of the testicular dysgenesis syndrome.

TA risk. $A1cf^{KO/+}$ heterozygosity reduced the risk for both TGCTs and testicular atrophy but not for cryptorchism, but with distinct PofO effects. Maternal $A1cf^{KO/+}$ heterozygosity affected atrophy risk in only one offspring genotype, whereas all progeny showed reduced TGCT risk (Table 2). Conversely, paternal $A1cf^{KO/+}$ heterozygosity affected the risk for atrophy in all progeny, whereas only one offspring genotype had reduced TGCT risk (Table 2). By contrast, $Ago2^{KO/+}$ heterozygosity increased the occurrence of cryptorchism, but not atrophy, only in intercrosses, whereas TGCT risk was reduced specifically in maternal $Ago2^{KO/+}$ backcrosses. Interestingly, as observed in humans (18, 21–23), a strong association was also found between cryptorchism and TGCTs in 129/Sv controls, with cooccurrence fourfold greater than independent occurrence (Table S2).

Reproductive performance. $A1cf^{KO/+}$ and $Ago2^{KO/+}$ heterozygotes had histologically normal gonads, although $A1cf^{KO/+}$ males had lower sperm counts, which have been associated with reduced fecundity (116). However, fertility was similar in *Ago2* test and 129/Sv control crosses. By contrast, the $A1cf^{KO}$ strain showed an increased reproductive performance with age and an increased litter index. Therefore, $A1cf^{KO/+}$ heterozygosity led to shortened litter intervals and extended reproductive lifespan.

TRD. Distorted genotypic transmission results in an atypical inheritance of specific genetic variants (117). The literature and our study reveal several RBPs, such as DND1, Pumilio1 (PUM1), and DEAD box helicase1 (DDX1) (53, 118, 119), in addition to A1CF and AGO2, which show TRD in mice (Table 3) (57, 61). TRD either favors ($A1cf^{KO}$, $Ddx1^{KO}$, $Pum1^{KO}$) or disfavors ($Ago2^{KO}$, $Dnd1^{KO}$) heterozygotes relative to wild-type (53, 57, 118, 119).

TRD may arise during gametogenesis, at fertilization, or during embryonic development, but in general the mechanisms are poorly understood (118). With rare exceptions, all ovulated eggs are fertilized. Therefore, the number of ovulated eggs, which is determined before mating, dictates litter size. For the $A1cf^{KO}$ and $Ago2^{KO}$ strains, complete embryonic lethality of homozygotes (57, 61) should reduce the litter sizes among intercrosses by 25% compared with backcrosses. However, the normal litter size in *A1cf* intercrosses suggests that genotype ratios differed significantly from Mendelian expectations without embryo loss of either wild types or heterozygotes. By contrast, the reduced litter size in *Ago2* intercrosses is consistent with the loss of homozygotes but not with reduced viability of heterozygotes; otherwise the average litter size for *Ago2* intercrosses would have been reduced by 50% compared with the backcrosses. Litter size and related measures of reproductive performance are not often reported but are essential for critically evaluating the consequences of genetic variants on meiosis, gametogenesis, and embryonic viability.

Conclusion

To ensure the viability and fertility of later generations, various molecular mechanisms monitor the germline for anomalies in DNA repair, DNA replication, cell-cycle control, and unpaired chromosomes (6, 120–123). Gametes must have the proper genetic constitution with few mutations or chromosome aberrations and appropriate epigenetic features (124, 125). Pluripotency must be rigorously controlled in the unipotent germline. When surveillance and pluripotency controls fail, infertility, embryonic lethality, gonadal dysgenesis, tumors, and TRD can ensue. Interestingly, many of these abnormalities are found in *Dnd1* (40, 53), *Pum1* (119, 126), *Ddx1* (118, 127), *Prdm9* (122), and *A1cf* and *Ago2* mutants (Tables 1–4). These genes, which encode factors controlling RNA availability for translation, reveal the essential role of RNA biology and epigenetics in fundamental aspects of germline surveillance.

Materials and Methods

The Pacific Northwest Diabetes Research Institute Institutional Animal Care and Use Committee approved all studies and procedures. We used two mutants, $A1cf^{KO/+}$ and $Ago2^{KO/+}$, and two kinds of crosses: reciprocal backcrosses to the 129/Sv wild-type mice and intercrosses. All killed males were examined for TGCTs and TAs (128). The χ^2 contingency and goodness-of-fit tests were used as appropriate to test relations between TAs, TGCTs, genotype, and paternal phenotype. Previously described methods were used to test for departures from Mendelian expectations of genotype segregation among intercross progeny (129). In all cases, the significance threshold was set at 0.05. To minimize the risk of false positives with multiple comparisons, we computed the false-discovery rate (FDR) (130), set at 0.1, for the six comparisons in each "gene family" of tests. Finally, fold-change was used as a measure of effect size. Emphasis was given to results that were statistically significant after estimation of the FDR and to some rare exceptions with strong effects (fold change >2). All methods are described in *SI Materials and Methods* and in refs. 131–133.

To control for substrain effects on the TGCT risk in the 129/Sv strain and mutant substrains, we backcrossed both mutants to 129/Sv and surveyed offspring for TGCTs over three generations (N1–N3). Any genetic difference between substrains that was not linked to either the $A1cf^{KO}$ or $Ago2^{KO}$ mutants should be lost at a rate of 0.5 per generation, with a probability of persisting over the three backcross generations in any given family line of 0.125. With multiple families for each mutant, the probability of a significant background effect is negligible. However, to test directly for possible substrain effects, we examined the occurrence of affected mice for each mutant for backcross generations N1–N3. The χ^2 contingency tests did not detect significant changes across generations (thresholds $P < 0.05$, FDR <0.1; see Table S7).

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