Mechanisms Underlying Mammalian Hybrid Sterility in Two Feline Interspecies Models

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Abstract

The phenomenon of male sterility in interspecies hybrids has been observed for over a century, however, few genes influencing this recurrent phenotype have been identified. Genetic investigations have been primarily limited to a small number of model organisms, thus limiting our understanding of the underlying molecular basis of this well-documented "rule of speciation." We utilized two interspecies hybrid cat breeds in a genome-wide association study employing the Illumina 63 K single-nucleotide polymorphism array. Collectively, we identified eight autosomal genes/gene regions underlying associations with hybrid male sterility (HMS) involved in the function of the blood-testis barrier, gamete structural development, and transcriptional regulation. We also identified several candidate hybrid sterility regions on the X chromosome, with most residing in close proximity to complex duplicated regions. Differential gene expression analyses revealed significant chromosome-wide upregulation of X chromosome transcripts in testes of sterile hybrids, which were enriched for genes involved in chromatin regulation of gene expression. Our expression results parallel those reported in *Mus* hybrids, supporting the "Large X-Effect" in mammalian HMS and the potential epigenetic basis for this phenomenon. These results support the value of the interspecies feline model as a powerful tool for comparison to rodent models of HMS, demonstrating unique aspects and potential commonalities that underpin mammalian reproductive isolation.

Key words: **hybrid sterility, Haldane's rule, large X-effect, speciation, feline.**

Article

Introduction

Reproductive isolation is a primary barrier supporting the establishment of new species by restricting gene flow between related taxa. Darwin recognized reproductive isolation as essential to the formation of nascent species. While examining hybridization, he observed a gradient of sterility and hypothesized a precursory causation of hybrid incompatibilities, stating that sterility was not a result of direct adaptation but was "incidental on other acquired differences" (Darwin 1859). In 1922, J.B.S. Haldane formalized hybridization observations from diverse organisms into what is now referred to as Haldane's Rule, which states that the heterogametic sex (XY males in mammals) is preferentially rare, absent, or sterile in the offspring of two different species (Haldane 1922). This phenomenon is observed across animal phyla, yet only a few genes that regulate hybrid sterility have been identified in two model organisms (i.e., *Drosophila* and mouse) (Sun et al. 2004; Mihola et al. 2009; Phadnis and Orr 2009). Dobzhansky (1937) and Muller (1942) proposed a simple model explaining hybrid male sterility (HMS) as the result of incompatible epistatic interactions (known as Dobzhansky–Muller incompatibilities) between divergent genomes. Few species have been formally utilized in genetic analyses to examine this principle of evolutionary biology and suitable vertebrate models with which to genetically dissect HMS are very rare. In mammals, a single mouse HMS locus has been identified: The autosomal H3K4 trimethyltransferase gene *Prdm9* (Mihola et al. 2009). Additionally, multiple regions of the mouse X chromosome have also been implicated (Storchová et al. 2004; Good et al. 2008; White et al. 2012), and while fine mapping has uncovered several candidate genes, none have been shown to be causal (Bhattacharyya et al. 2014).

The observation that the X chromosome harbors many loci underlying HMS underpins one of the rules of speciation (Coyne and Orr 2004), the "Large X-Effect," based on its disproportionately large influence on postzygotic reproductive isolation. In *Drosophila*, genomic incompatibilities are enriched on the X and heterospecific introgression of the X induces sterility more readily than autosomes (Turelli and Orr 1995; Masly and Presgraves 2007). In natural populations, gene flow is drastically reduced on the X chromosome compared with autosomes (Macholán et al. 2007). Multiple mechanisms have been proposed to explain this phenomenon, including "Dominance theory," which is based on the exposure of recessive epistatic X-linked incompatibilities in hemizygous males (Turelli and Orr 1995), and "Faster-male theory," which suggests male-limited reproductive traits accumulate

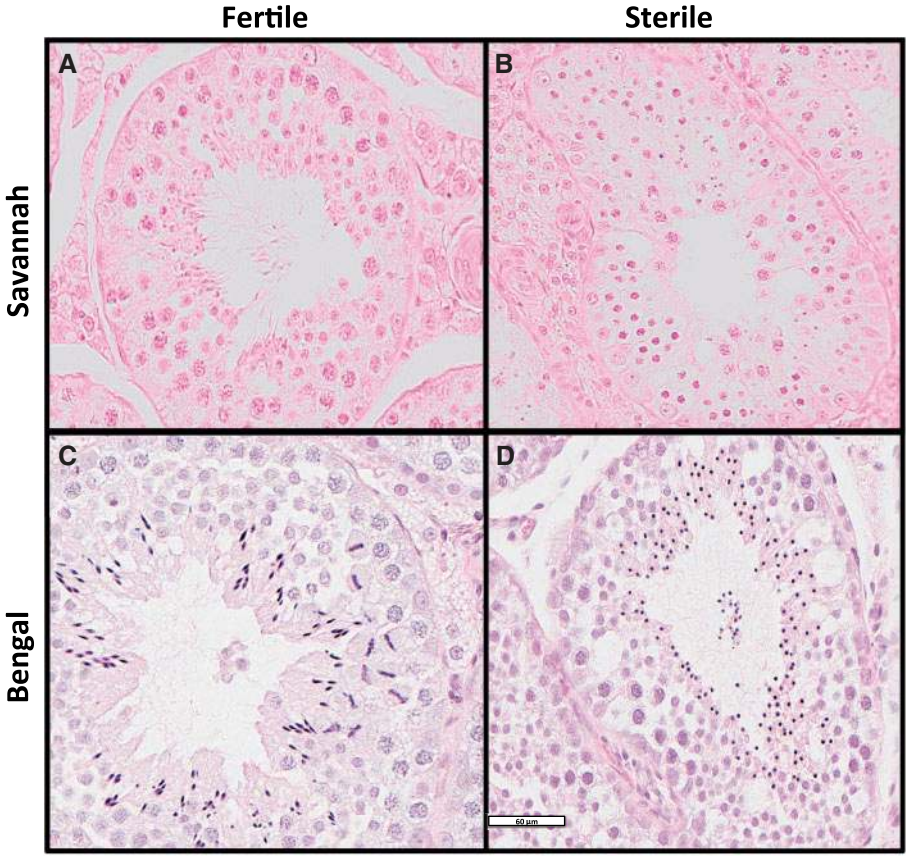


FIG. 1. H&E-stained testis from fertile and sterile hybrids. (*A*) Fertile fifth-generation Savannah backcross hybrid testis with mature, normal sperm. (*B*) Sterile fourth generation Savannah backcross hybrid testis showing hypospermatogenesis, with a high incidence of pachytene spermatocytes and the presence of vacuoles. (*C*) Fertile fourth-generation Bengal backcross hybrid testis with mature, normal sperm. (*D*) Sterile fourth-generation Bengal backcross hybrid testis exhibiting globozoospermia.

incompatibilities faster than do female-limited traits, due either to the sensitivity of spermatogenesis to molecular perturbation and/or to sexual selection (Wu and Davis 1993). More generally, the X may simply evolve at a faster rate than the autosomes ("faster-X theory") and accumulate incompatibilities more readily (Charlesworth et al. 1987). Mouse models support the Large X-Effect based on results from natural hybrid zones (Macholán et al. 2007) and experimental crosses (Good et al. 2008, 2010; White et al. 2012; Bhattacharyya et al. 2014). Given the rapid evolution of reproductive genes and pathways, it currently remains unclear, however, whether the same or even convergent genetic mechanisms or pathways drive HMS in divergent taxa.

Reproductive isolation via HMS is proposed to be polygenic, involving genes with both large and small influence (Turelli and Orr 1995; Maside and Naveira 1996). Interspecies crosses are powerful resources for mapping complex traits and examining the mechanisms of speciation, requiring fewer individuals to map genes with moderate to large phenotypic effects (L'Hôte et al. 2010). To explore the genetic basis of Haldane's Rule and the potential for a Large X-Effect in a novel, complimentary mammalian model, we exploited two interspecies cat hybrids that are among the most common cat breeds worldwide. Despite a 10 My evolutionary divergence time (Li G, Davis BW, Eizirik E, Murphy WJ, submitted), domestic cats will naturally interbreed with African servals (*Profelis serval*) in captivity to produce the Savannah breed, with over 10,500 registered members worldwide (Wheeler 2011). A second intercross between domestic cats and Asian leopard cats (*Prionailurus bengalensis*) produces the Bengal, one of the most popular cat breeds in the world (Johnson 1991). These latter two species diverged approximately 7.2 Ma (Li G, Davis BW, Eizirik E, Murphy WJ, submitted).

The foundation lines for each breed almost exclusively originate through mating female domestic cats to males of the wild species. Fertilization may be accomplished by natural mating, or more rarely, via artificial insemination. Lines are normally perpetuated via unidirectional crossing of fertile F1 females to male domestic cats. Hybrid sterility manifests similarly in both breeds with F1 and early backcross generations exhibiting azoospermia and severe seminiferous tubule degeneration. Later backcross generations show meiotic progression but still may produce low amounts of sperm with a high proportion of abnormalities (fig. 1*B* and *D*). Later generation hybrid cats show fertility characteristics analogous to normospermic domestic cats (fig. 1*A* and *C*); however, on average, Bengal males regain fertility in earlier backcross

generations than do Savannahs (supplementary fig. S1, Supplementary Material online). This is complicated as late generation, fertile Bengal and Savannah males are often used to perpetuate breeding lines via intercrossing with fertile F1 or backcross females from early generations.

Here, we demonstrate the utility of these hybrid cat models for detecting loci that contribute to HMS by genotyping cohorts of each hybrid breed on the Illumina 63 K domestic cat single-nucleotide polymorphism (SNP) array. Following the Dobzhansky–Muller model, we hypothesize that sterile individuals will have an enrichment for SNP alleles putatively descending from the wild progenitor species rather than of domestic origin. We also present genome-wide expression results based on testis RNAseq data from the parent species and fertile and sterile hybrid backcross cats, to investigate patterns of genome-wide and locus-specific misexpression associated with HMS.

Results

Genome-Wide Association Study Identifies Multiple Candidate Hybrid Sterility Genes in Savannahs and Bengals

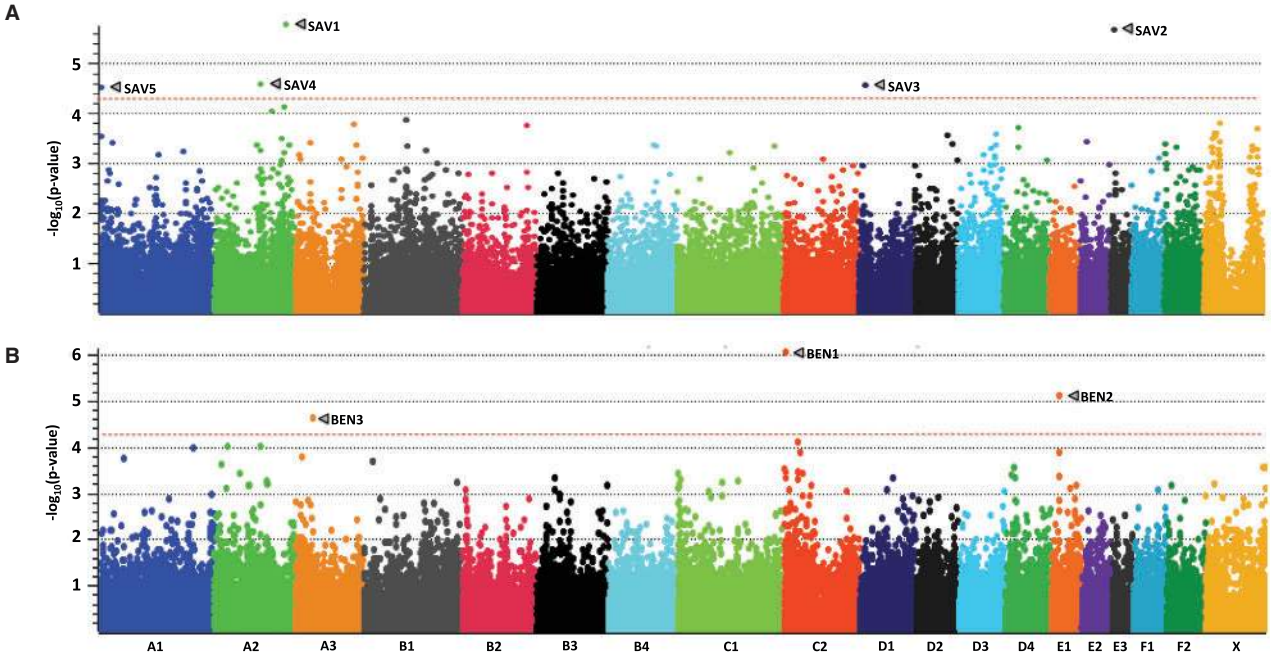
We analyzed cohorts of sterile and fertile Bengals and Savannahs in independent genome-wide association studies (GWAS) to identify SNPs associated with the sterility phenotype. We leveraged the domestic cat SNP array to detect markers fixed in wild progenitor species but variable in the domestic cat. The results of our two GWAS produced biologically relevant associations for five chromosomal regions in Savannahs and three in Bengals (fig. 2). For seven of the eight loci associated with sterility, there was an enrichment of 1) putative ancestral alleles that were monomorphic in the wild parent species and 2) homozygous wild genotypes in sterile hybrids (supplementary fig. S2, Supplementary Material online). This enrichment for wild alleles within a predominantly domestic cat genomic background follows the Dobzhansky–Muller model and may drive reproductive isolation by genomic incompatibility, thereby corroborating the theoretical predictions of our model system.

In the savannah GWAS, two top ranking SNP markers mapped within a pair of genes (*CADM1* and *AKAP9*, fig. 3*A* and *B*) that promote proper organization and function of the blood-testis barrier (BTB), which is vital for germ cell development and sperm quality by influencing the ultrastructural composition of developing sperm (Cavicchia et al. 1996). Both genes are essential to spermatogenesis in mouse and are upregulated in testes of sterile Savannahs (fig. 3*A* and *B*). CADM1 serves as an adhesion molecule at the Sertoli-spermatid interface of the BTB, and *Cadm1*$^{-/-}$ mouse mutants show oligoastheno-teratozoospermia and BTB disruption (Maekawa et al. 2011). Like *Cadm1*, *Akap9* null mice demonstrate failure of spermatogenic progression due to mislocalization of developing germ cells (Schimenti et al. 2013). Similar phenotypes are observed in some sterile savannahs (fig. 1*B*, supplementary fig. S3, Supplementary Material online). *AKAP9* is unique among all the identified candidate HMS genes in that it is the only gene to display significant nonsynonymous evolution between the domestic cat and wild species (supplementary table S1, Supplementary Material online). All other genes show few or no amino acid altering substitutions that would be predicted under the faster-male hypothesis, thus implying a major role for interspecific regulatory divergence in feline HMS (Good et al. 2010).

The top-ranking marker in the Savannah GWAS (additive model) lies within intron 4 of *GRM8* (glutamate receptor, metabotropic 8). This is the only testis-expressed gene within one megabase of the associated marker and is significantly downregulated in testes of sterile Savannahs (fig. 3*C*). In mice, Grm8 is present on the surface of spermatozoa and neurons (Lee et al. 2011). Although its precise function within spermatogenesis is currently unknown, GRM8 is one of the most highly expressed metabotropic glutamate receptors in the human testis (Julio-Pieper et al. 2013). The fourth significant savannah SNP resides within *LATS2*, which encodes a novel multifunctional serine/threonine kinase whose roles include control of cell proliferation and ensuring accurate ploidy during cell division (McPherson et al. 2004). This gene induces G2/M arrest and apoptosis via its kinase activity (Kamikubo et al. 2003) and is significantly downregulated in sterile Savannah testes (fig. 3*D*). The fifth SNP localizes to a gene-dense region on chromosome E3 that contains a zinc-finger gene cluster and resides within *ZSCAN25*, a gene that encodes a SCAN domain-containing transcription factor of unknown function (fig. 3*E*).

In the Bengal cohort analysis, the top sterility-associated SNP marker resides within an intergenic interval between two genes (fig. 3*F*). Immediately upstream of the SNP lies DNA methyltransferase 3-like (*DNMT3L*), which encodes a product that regulates the establishment of DNA methylation at dispersed and paternally inherited sites in the early stages of spermatogenesis (Bourc'his et al. 2001). *Dnmt3l-/-* male mice are sterile due to failure of meiosis as a result of hypomethylation (Webster et al. 2005) and exhibit phenotypes similar to those of many sterile Bengal hybrids, including loss of spermatocytes due to sloughing, vacuolarization, and a Sertoli-cell-only like appearance (fig. 1*D*, supplementary fig. S3, Supplementary Material online). *DNMT3L* normal expression and relative misexpression was the highest of any gene in the candidate gene region. Moreover, misregulation patterns were in opposing directions depending on the parent species under comparison (i.e., domestic vs. wild). *DNMT3L* was upregulated in sterile hybrids relative to domestic cats and fertile hybrids but downregulated in comparisons with the Asian leopard cat, suggesting divergent levels of transcription that may be incompatible in a hybrid background due to dosage effects (fig. 3*F*). Downstream of the same top ranking SNP lies *C21orf33*, a mitochondrial homolog of *HES1*. Although no specific function has been ascribed to this gene, the protein product is decreased in sperm samples from men with unexplained infertility issues (McReynolds et al. 2014) and may warrant future consideration.

Three remaining Bengal GWAS markers reside within or near a pair of genes whose products putatively interact



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GWAS Marker	-log10(P-value) Additive / Dominant	felCat5 Coordinates	Marker Location Relative to Proximal Expressed Gene
SAV1 (A2.226531983)	**5.819** / 4.127	A2: 143,613,066	*GRM8*, intron
SAV2 (E3.7948479)	4.768 / **5.691**	E3: 6,557,367	*ZSCAN25* exon
SAV3 (D1.15940780)	4.239 / **4.578**	D1: 12,989,298	*CADM1*, Intron
SAV4 (A2.153566027)	4.093 / **4.586**	A2: 95,948,859	*AKAP9*, Intron
SAV5 (A1.2074489)	2.725 / **4.533**	A1: 1,959,47	*LATS2*, Intron
BEN1 (C2.2183337)	5.646 / **6.069**	C2: 2,277,174	*DNMT3L*, downstream
BEN2 (E1.41343439)	3.100 / **5.117**	E1: 15,300,704	*VPS53*, intron
BEN3 (A3.41694977 / Un.19202403)	4.171 / **4.641**	A3: 35,837,793 / 35,863,311	*SNAP25*, upstream

Fig. 2. Manhattan plots and marker details for GWAS in two hybrid feline breeds. (*A*) Five markers (SAV1-5) exceeding Wellcome Trust recommendations for genome-wide significance ($P_{uncorrected} < 5 \times 10^{-5}$; $-\log_{10} P = 4.30$, red line) (Wellcome Trust Case Control Consortium 2007) based on analysis of the savannah cohort ($n = 103$). The Manhattan plot shown represents the full results under the dominant model of inheritance, but for brevity, we inserted the −log10 *P* value of SAV1 based on the additive model, for comparison. The full Manhattan plot under the additive model is shown in supplementary figure S9, Supplementary Material online. (*B*) Manhattan plot under a dominant model of inheritance for the Bengal cohort ($n = 101$), showing three markers (the two markers for BEN3 are in LD) exceeding genome-wide significance (red line) (Wellcome Trust Case Control Consortium 2007). (*C*) Table of markers, *P* values, coordinates in the FelCat5 assembly and most proximal gene to each marker. P-P plots for each analysis are shown in supplementary figure S10, Supplementary Material online.

and are involved in intracellular trafficking via the Golgi-associated retrograde protein (GARP)/SNARE complex, a process that is essential for proper sperm development. The product of the first gene, *VPS53*, cooperates with VPS52 and VPS54 to form the GARP complex (Hickey et al. 2013). Defects in GARP lead to acrosome biogenesis failure and globozoospermia (Jockusch et al. 2014), a phenotype also observed within some sterile Bengals (fig. 1*D*). The second gene, *SNAP25*, possessed two significant markers approximately 25–50 kb upstream of the transcriptional start site. SNAP25 is a component of the SNARE complex, which directly interacts with the GARP complex (Fasshauer and Margittai 2004). *SNAP25* was moderately upregulated in sterile versus fertile hybrids (fig. 3*G*). The VPS53 amino acid sequence is conserved between the domestic cat and Asian leopard cat, and testis transcription showed only modest changes in expression between sterile hybrids and domestic cats but more pronounced differences with the Asian leopard cat (fig. 3*H*). Taken together, these observations lead us to hypothesize that misregulation of both genes may alter critical GARP-SNARE interactions that support proper acrosomal formation.

X Chromosome-Wide Overexpression in Hybrid Testes

Whole-testis transcriptome RNAseq data showed very similar expression patterns between Bengal and Savannah hybrids: 70% of all misregulated transcripts were shared between both hybrid breeds when sterile hybrids were compared with domestic cat or fertile hybrids (supplementary fig. S4, Supplementary Material online) and measures of misregulation were robust to different normalization methods (supplementary fig. S5, Supplementary Material online). We observed a very strong and statistically significant skew toward upregulation of the X chromosome relative to that of autosomes, in both sterile Savannahs and Bengals (fig. 4*B* and *C*) (Komolgorov–Smirnov $P < 0.01$). Furthermore, only the X

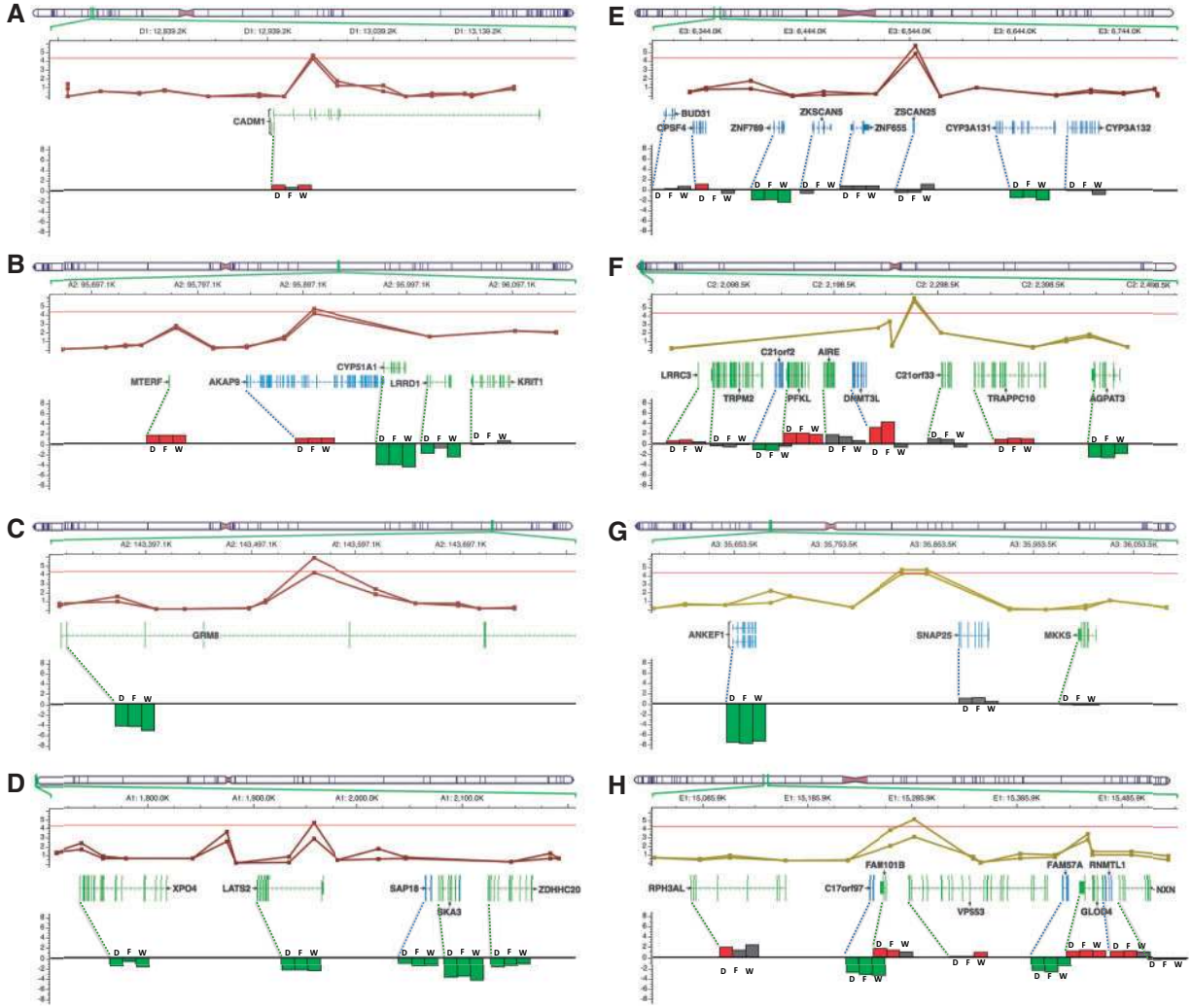

Fig. 3. (*A–H*) Genomic regions surrounding each top ranked marker and testis expression data for the region. A 500-kb window surrounding each significant marker is indicated by vertical lines on the chromosome ideogram. –log10 *P* values (*y* axis) for both additive and dominant inheritance models are plotted along each chromosome (*x* axis), with significance threshold indicated by horizontal red line. Log_2 fold testis expression change in sterile hybrids when compared with domestic cat (D), fertile hybrids (F), and wild species (W) are shown in red (significant upregulation), green (significant downregulation), and gray (nonsignificant misregulation) (supplementary table S8, Supplementary Material online).

chromosome was found to possess significant chromosome-wide misexpression between sterile and fertile individuals (Bengals: $P = 5.0 \times 10^{-35}$, Savannahs: $P = 1.5 \times 10^{-22}$, supplementary table S2, Supplementary Material online). This same X-upregulation bias was observed in laboratory mouse intraspecies hybrids (Good et al. 2010; Campbell et al. 2013), and we identified a suite of shared genes misregulated in both rodent and feline hybrids (supplementary table S3, Supplementary Material online). An analysis of functional gene enrichment of the upregulated feline X chromosome genes revealed "chromatin regulation" as the top enriched category for both Bengal and Savannah hybrids (supplementary table S4, Supplementary Material online).

One complication in assessing gene expression differences between the developmentally different testes of sterile versus fertile animals is the "cellular composition" bias (Good et al. 2010). Specifically, postmeiotic cells are found in lower proportions in sterile testes, therefore their transcripts are expected to show lower expression levels compared with normal testes, even if transcript levels were equivalent per cell in the two samples. As a result, it has been proposed that in sterile testes, postmeiotic genes will appear to have reduced expression levels, whereas mitotic cells will appear overexpressed, and therefore differential expression may not actually reflect true misregulation. To examine whether the proposed tissue composition bias impacted our differential expression results, we examined the misregulation patterns of three genes that were shown to be robust to tissue composition in comparisons between normal human adult testis and multiple testicular germ cell tumor pathologies (Svingen et al. 2014) and have been validated to be reliable for normalization experiments. Two of these putative "housekeeping" genes, *RPS20* and *SRSF4*, were not misregulated in any of the sterile hybrid to fertile comparisons (supplementary table S5, Supplementary Material online). The third gene, *RPS29*, was not examined due to the presence of multiple processed pseudogenes and numerous high identity BLAT hits elsewhere in the domestic cat genome, making it a poor

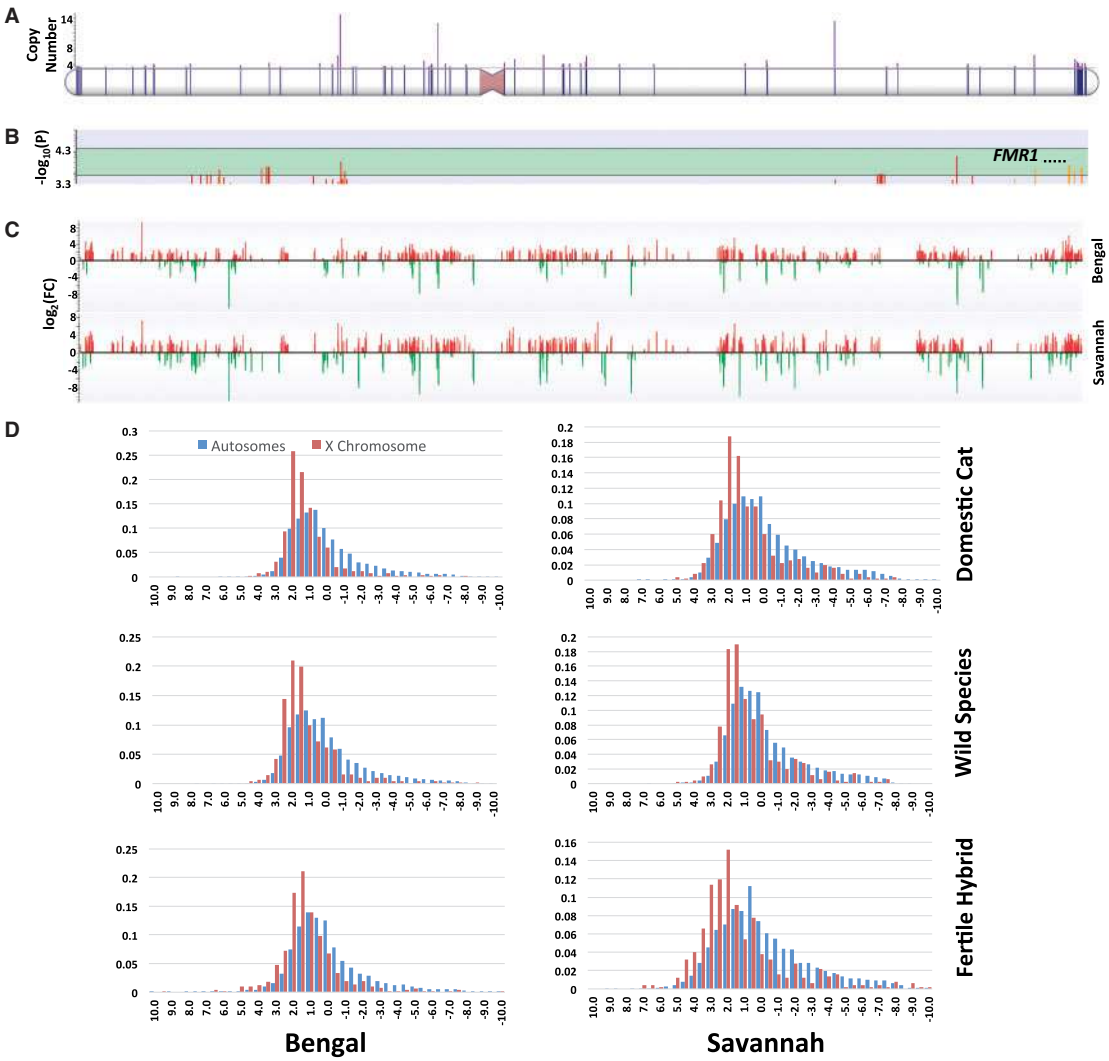

FIG. 4. X chromosome copy number, GWAS significance, and RNA-seq gene misregulation in sterile feline hybrids. (*A*) Number of duplicated copies detected by CNVator across the X chromosome. Blue lines on the ideogram indicate position along the X chromosome (*x* axis). Purple lines indicate number of detected copies (*y* axis). (*B*) $-\text{Log}_{10}$ *P* values of SNP markers (only values above 3.3 are shown, for brevity) that approached significance on the X chromosome of each breed. Orange vertical lines indicate Bengal and red indicates Savannah. (*C*) Gene misexpression on the X chromosome in each sterile hybrid when compared with the domestic cat. Red indicates upregulation and green downregulation. The *x* axis denotes the position along the X chromosome, and the *y* axis indicates the $\log_2$ (fold change) in expression for each gene when compared with domestic cat. (*D*) Contrasting misregulation patterns on the X chromosome and autosomes in sterile hybrids when compared with domestic cat, fertile hybrids, and wild species. *x* axes list the integer fold-change categories (from negative 10-fold change to positive 10-fold change) for which each gene is classified based on the magnitude of misexpression. *y* axes show the frequency of genes falling into these categories. Each X-autosome distribution was significantly different for all six comparisons (Komolgorov–Smirnov $P < 0.01$).

candidate for RNAseq normalization. The stability of *RPS20* and *SRSF4* across fertile and sterile testes suggests that tissue composition is unlikely to have strongly biased our results, specifically the highly significant chromosome-wide upregulation of genes on the X chromosome and not on any autosome (supplementary table S2, Supplementary Material online). Future experiments using flow-sorted testis cell-type preparations will further test the validity of these results.

X-chromosome overexpression in hybrid mice is hypothesized to be due in part to failed trans-acting loci on the X chromosome (Good et al. 2008; White et al. 2012; Turner et al. 2014). GWAS results for the X chromosome identified several suggestive markers ($P_{\text{uncorrected}} < 5 \times 10^{-4}$) that approached but did not exceed our imposed significance threshold (see Materials and Methods; supplementary table S6, Supplementary Material online). We attribute this to 1) lower X chromosome SNP density on the array (supplementary fig. S6, Supplementary Material online) and 2) the observation in less divergent mouse intraspecific hybrids that the Large X-Effect is polygenic (Good et al. 2008). Given the greater evolutionary divergence observed in the feline interspecific hybrids, we hypothesized that the X-linked signal would be even more polygenic, with multiple X-linked loci modulating small to moderate individual effects. Nevertheless, in Savannahs, 42% of the top 50 SNP markers were X-linked, thereby affirming an X-linked contribution to

sterility (supplementary table S7, Supplementary Material online). When we compared the highest X-linked GWAS regions defined by SNPs in Savannahs and Bengals (fig. 4*B*, supplementary table S6, Supplementary Material online) to regional duplication profiles generated by structural variation analysis, we observed that most of the top markers mapped near regions containing duplications (fig. 4*A*).

In the Bengal GWAS, the top four X-linked markers shared identical support values ($-\log_{10} P = 3.57$), and one marker maps 70 kb upstream of fragile X mental retardation syndrome 1 homolog (*FMR1*), a highly expressed gene in mouse spermatogonia and spermatocytes (Hickey et al. 2013; Alpatov et al. 2014). Notably, *Fmr1* resides within the mouse *Hstx2* (hybrid sterility X 2) critical interval (Bhattacharyya et al. 2014). A cluster of three additional SNP markers localize to distal Xq within another structurally complex region (fig. 4*A*). In humans, naturally occurring duplications within the syntenic Xq27.3–q28 region induce fertility problems and reduce testis weight, underpinning the role of this region in eutherian mammal testis function.

Discussion

As an increasing number of intra and interspecific genome comparisons become available, an emerging theme from these studies is that postspeciation gene flow often extends for several million years following an initial divergence (Toews and Brelsford 2012; Kutschera et al. 2014; Li G, Davis BW, Eizirik E, Murphy WJ, submitted). Although our examination of divergent interspecies hybrids cannot guarantee identification of all genomic regions that may have initiated the divergence of these felid species, it does highlight several that are essential to the reproductive success of males by their disruption in hybrids and hence, those potentially restricting postspeciation gene flow. Our combined GWAS plus RNAseq strategy identified multiple candidate hybrid sterility genes and a strong X-linked expression bias in sterile hybrids. Collectively, our results implicate several molecular/cellular pathways that appear to play a major role in felid hybrid sterility, as well as some intriguing commonalities with mouse models of hybrid sterility that may point to conserved mammalian pathways that are sensitive to hybrid incompatibilities.

BTB Compartmentalization Failure

Regulation of cellular signaling between developing germ cells and supporting Sertoli cells is necessary for accurate spermatogenesis in mammals. Proper organization of the BTB is essential to this process (Mital et al. 2011). The primary function of adult Sertoli cells is to provide structure to the seminiferous tubule, nurture maturing germ cells, and compose specialized junction complexes that comprise the BTB (Mruk and Cheng 2004). One of these specialized junctions is the ectoplasmic specialization (ES). Assembly of the ES anchors elongating spermatids to Sertoli cells via the forming acrosome and disassembly releases sperm into the seminiferous tubule lumen (Vogl et al. 2000). *CADM1* is a Sertoli-spermatid adhesion molecule in the apical ES, and *Cadm1* deficiency causes round and elongating spermatids to detach from Sertoli cells, resulting in sloughing of germ cells prior to the completion of maturation (Wakayama et al. 2007; Wakayama and Iseki 2009). The resulting severe sperm head and midpiece abnormalities cause poor binding to the zona pellucida (Mueller et al. 2003). These phenotypes are similar to those observed in hybrid cats (fig. 1*B* and *D*, supplementary figs. S2 and S7, Supplementary Material online).

AKAP9 also plays a role in the BTB, and when ablated in mice, the BTB junction organization is disrupted, leading to compartmentalization failure of developing spermatozoa by Sertoli cells (Cavicchia et al. 1996), which is characterized by mislocalization of developing germ cells, few round spermatids, and Sertoli cells with characteristics resembling prepubertal testes (Sehrawat et al. 2011). The large sequence divergence between domestic cat and serval *AKAP9* alleles in sterile Savannah hybrids, including putative frameshift and numerous nonsynonymous substitutions, may facilitate the severe testis compartmentalization and failure of sperm production present in the Savannah HMS phenotype (fig. 1*B*, supplementary fig. S3, Supplementary Material online). AKAP9 also functions as a scaffolding protein that is required for assembling phosphatases and protein kinases on the centrosome and Golgi apparatus. It is therefore plausible that AKAP9 interacts with another gene product identified in our GWAS, the serine/threonine kinase LATS2, which localizes to centrosomes and regulates the cell cycle and apoptosis (Yabuta et al. 2014).

Acrosomal Development and GARP/SNARE Intracellular Trafficking

The importance of acrosome development in felid HMS phenotypes is highlighted by three of the top eight genes in the two separate GWAS, including the top ranked Savannah GWAS marker that resides within *GRM8*. Multiple lines of evidence point to a role for GRM8 in sperm acrosome function (Lee et al. 2011; Julio-Pieper et al. 2013) and knockout of a similar gene, *Gmr7*, produces mice with reduced numbers of motile sperm (Marciniak et al. 2014). One clue to the role of *GRM8* in spermatogenesis and hybrid sterility is the cAMP regulatory task played by GRM8 in other tissues. Because spermatozoa control the acrosome reaction via cAMP-dependent processes (Lin et al. 2006), the sperm-specific presence of this mGlu receptor in the mammalian acrosome may repress the production of cAMP, and in turn prevent acrosome exocytosis (Baker et al. 2013). Therefore, the observed downregulation of this gene in sterile hybrid testes is consistent with a failed interaction within a hybrid background.

Cargo transport between endosomal-lysosome membranes is essential to the proper development of the sperm acrosome (Vassilieva and Nusrat 2008). Vesicular membrane fusion is a vital component of sperm maturation that requires interaction of the SNARE motif and the N-terminus of VPS53/VPS54 within the GARP complex (Pérez-Victoria and Bonifacino 2009). A naturally occurring missense mutation in *VPS54*, the co-interacting partner of our candidate gene *VPS53*, defines the "wobbler" mouse mutant, and changes the

dynamics of retrograde endocytosis between the sperm head and the Golgi, impairing acrosome formation and inducing globozoospermia (Paiardi et al. 2011). Furthermore, the weakening of Sertoli-germ cell contacts in "wobbler" mutants impedes the promotion of germ cell survival and maturation in a manner similar to BTB failure, resulting in poor motility and overall reduced germ cell numbers. Both phenotypes are observed in sterile Bengal hybrids (fig. 1*B*, supplementary fig. S3, Supplementary Material online). SNAP25 is a primary component of the SNARE complex and is essential to acrosome exocytosis (Tomes et al. 2002). Given the demonstrated link between GARP-SNARE complexes in the formation of the acrosome, we hypothesize that VPS53 and SNAP25 represent partners in a Dobzhansky–Muller incompatibility in the Bengal interspecies model and warrant further study within the contexts of normal spermatogenesis and reproductive isolation.

Transcriptional Regulation and Methylation

One emerging feature shared by proteins encoded by *Drosophila* and mouse HMS genes, as well as genes found within chromosomal regions of high intraspecific divergence, is that they encode binding proteins involved in transcriptional regulation (Carneiro et al. 2014). The putative functions of several candidate felid HMS genes identified in this study as well as the observed patterns of gene misregulation also suggest an important role of transcriptional regulation in feline HMS (Good et al. 2010). For example, the second ranked Savannah HMS SNP resides within *ZSCAN25*, which encodes a zinc-finger protein with SCAN domains important for DNA binding and transcriptional regulation. ZSCAN25 is one of many mammalian transcription factors with unknown function, and our results warrant further functional investigation via knockdown strategies to better understand its role in normal spermatogenesis. Notably, a mouse hybrid sterility QTL localizes to this same gene-dense interval on the distal q-arm of mouse chromosome 5 and also overlaps a strong expression QTL (White et al. 2012; Turner et al. 2014). Further fine mapping of both feline and mouse loci would address the intriguing possibility of an HMS gene shared across mammalian orders.

The top ranked Bengal GWAS SNP lies between two genes, *DNMT3L* and *C21orf33*, neither of which could be excluded based on our existing mapping resolution or RNAseq analyses. However, the most compelling HMS candidate gene is *DNMT3L*, based on several key observations. DNMT3L regulates the functional activity of two DNA methyltransferases, DNMT3A/B, which are required during early meiotic stages of development, and its absence leads to a myriad of meiotic defects and male infertility (Bourc'his et al. 2001; Webster et al. 2005). Many of these meiotic defects were also observed in testes of sterile Bengal hybrids (supplementary fig. S3, Supplementary Material online). *Dnmt3l* also resides within a mouse hybrid sterility QTL/eQTL region on chromosome 10 (Turner et al. 2014), and its downstream regulatory target *Dnmt3a* is misregulated in mouse hybrids (Good et al. 2010). DNMT3L regulates chromatin changes essential for formation of the sex body during the pachytene stage of meiosis that silences sex chromosome gene expression, a process termed meiotic sex chromosome inactivation (Burgoyne et al. 2009). *Dnmt3L* heterozygous mutant male mice show extended sex bodies and misregulation of a large number of meiotic transcripts, many from the X chromosome (Zamudio et al. 2011). This known connection between *Dnmt3L* function and X chromosome epigenetic modifications is strongly suggestive of a connection between this candidate hybrid sterility locus and the overexpression observed in X chromosomes of sterile Bengal hybrids. Finally, the concordant pattern of testis misregulation observed in the two feline interspecies hybrids (supplementary fig. S4, Supplementary Material online) indicates a similar mechanism influencing male sterility within both felid hybrids.

Conservation of the Large X-Effect in Mammalian Hybrid Gene Expression

The role of the X chromosome in hybrid dysfunction is well documented across animal phyla, but the molecular basis of the Large X-Effect in different organisms is not fully understood. Any number of properties of the X chromosome, individually or in combination, may contribute to the Large X-Effect, including faster male evolution (Wu and Davis 1993; Torgerson and Singh 2003), adaptive evolution of male-biased genes (Ellegren and Parsch 2007), and gene movement (Moyle et al. 2010). Moreover, patterns in X chromosome-wide overexpression in sterile intraspecific mouse hybrids (Good et al. 2010; Turner et al. 2014) allude to the influence of both cis- and trans-acting factors on the Large X-Effect. The enrichment of X-upregulated genes involved in chromatin modification in felid hybrids coupled with the disruption of meiotic sex chromosome inactivation in infertile hybrid mice (Good et al. 2010; Campbell et al. 2013; Bhattacharyya et al. 2014) indicates the plausibility of either conserved or convergent regulatory mechanisms shared between felid and rodent hybrids.

One of four top ranked Bengal X-linked SNP markers ($-\log_{10} P = 3.57$) lies immediately upstream of *FMR1*, which encodes fragile X mental retardation protein FMRP. FMRP has been shown to interact with pachytene-stage meiotic chromosomes and DNA damage response components during mouse spermatogenesis, and *Fmr1* knockout mice show apoptosis and failure of spermatogenesis (Alpatov et al. 2014). FMRP's direct chromatin interactions with components necessary for sex body formation suggest a potential role in hybrid X misregulation, especially when considered within the context of the misregulated genes associated with chromatin binding in sterile cat hybrids. Given that the top ranked Bengal autosomal candidate gene *DNMT3L* directly regulates methylation and epigenetic changes in early spermatogenesis, and histone H3K79 methylation aids in recruitment of FMRP to meiotic chromatin, we speculate that failed heterospecific interactions involving DNMT3L and FMRP may contribute to the X chromosome-wide upregulation that induces apoptosis and sterility.

In addition to the region upstream of *FMR1*, we identified several other candidate X chromosome intervals potentially associated with the sterility phenotype in both hybrid crosses. Interestingly, many of the top X chromosome SNPs reside adjacent to or within structurally complex chromosomal regions that contain duplications (fig. 4*A* and *B*). This observation is relevant based on the previously described association between mapped mouse hybrid sterility QTL and ampliconic gene clusters on the mouse X chromosome (Mueller et al. 2013). In mice, ampliconic gene clusters are enriched for postmeiotic genes and comprise 18% of all protein-coding genes on the mouse X (Mueller et al. 2008). These highly repetitive regions are typically collapsed and/or unassigned in the majority of draft mammalian genome assemblies, like cat, but leave signatures of high copy number.

The recent resolution of mouse X and Y chromosome amplicon sequences has led to the recognition of several gene family members that are shared between the two sex chromosomes and whose relative copy number has evolved under the pressures of sex-linked meiotic drive (Soh et al. 2014), an additional mechanism that has been proposed to underlie the Large X-Effect. Two particular genes, *Sly* on the Y chromosome and *Slx* on the X chromosome, are among the largest genes families, with greater than 130 and 25 functional copies in the *Mus musculus* C57BL/6 strain, respectively. Mice lacking adequate *Sly* expression, either via chromosomal deletion or knockdown show sperm abnormalities and infertility (Touré et al. 2004; Ellis et al. 2005; Cocquet et al. 2009). In both scenarios, the paralogous *Slx* gene(s) and other postmeiotic genes are upregulated. This has led to the hypothesis that *Slx* and *Sly* recently evolved in the genus *Mus* as a meiotic drive system and that the acquisition and massive amplification of these ampliconic gene families has been driven by X-Y interchromosomal conflict (Cocquet et al. 2009; Ellis et al. 2011; Soh et al. 2014).

X-Y conflict may be widespread in mammals, as evidenced by lineage-specific X-Y shared gene families in several species, including *VCX-VCY* gene families in simian primates (Skaletsky et al. 2003) and *CUL4BX-CUL4BY* in the cat family (Murphy et al. 2006; Li et al. 2013). Felid ampliconic Y chromosome gene families show considerable size and sequence variation between different cat species and therefore may have evolved as a parallel genomic manifestation of sex-linked meiotic drive (Li et al. 2013). We hypothesize that copy number variation between sex-linked amplicons of different species may provide a mechanism for reproductive isolation and the "islands of speciation" that are commonly observed in mammalian hybrid zones (e.g., Carneiro et al. 2014; Phifer-Rixey et al. 2014). Future fine mapping using unbiased, sequence-based approaches in larger cohorts will aid in the further elucidation of specific X-linked genes/gene families that contribute to feline HMS and allow us to test this hypothesis.

In summary, we identified eight autosomal HMS candidate loci and several X chromosome candidate regions within hybrids of two divergent feline interspecific crosses. Most HMS loci relate to a single compelling hybrid sterility candidate gene based on mapping resolution, expression data, and published functional data for mouse and human. At least three candidate gene regions are syntenic with mouse hybrid sterility QTL/eQTL on Chromosomes 5, 10, and X, raising the possibility that conserved hybrid sterility network components might be disrupted in mouse and cat hybrids. Felid X chromosome genes were strongly overexpressed in sterile hybrids relative to fertile controls and were functionally enriched for chromatin-modification-related biological processes, suggesting a similar epigenetic manifestation of the Large X-Effect in both felids and mice. Divergence of noncoding regulatory sequences has long been suspected to play an important role in interspecies divergence (King and Wilson 1975; Haerty and Singh 2006), and this point is emphasized by the lack of amino acid divergence, yet significant expression divergence and hybrid misregulation observed for most felid HMS genes. Future resequencing and functional/epigenetic annotation of candidate intervals defined by these two hybrid crosses will shed light on the mechanistic basis of noncoding incompatibilities that lead to HMS. Finally, similar genetic mapping studies in less-diverged feline interspecific breeds, such as the Chausie (a hybrid between the Jungle cat and domestic cat, which diverged ~3–4 Ma) will expand opportunities to examine the evolutionary origins and accumulation of hybrid incompatibilities within this recent species radiation.

Materials and Methods

Creation of Interspecies Domestic Cat Hybrids

To produce F1 Savannah and Bengal hybrids, breeders selected males from African serval and Asian leopard cat species, respectively. These were crossed to female domestic cats by either cohabitation and natural mating or more rarely, artificial insemination. Testes of F1 males from both crosses show Leydig cell hyperplasia with seminiferous tubules characterized by Sertoli-cell-only phenotype and an absence of a defined lumen (supplementary fig. S8, Supplementary Material online). First-generation backcross males generally show a similar, though often milder phenotype, with the occasional presence of spermatogonia and early spermatocytes. F1 hybrid females from both breeds are fertile, thus they are used in subsequent, unidirectional backcross matings to fertile domestic cat males, until fertile backcross hybrid males are produced. The backcross generation in which each hybrid breed regains fertility varies with evolutionary distance between the pair of parent cat species (over 10 and 7 My for the parents of the Savannah and Bengal, respectively) (Li G, Davis BW, Eizirik E, Murphy WJ, submitted). Within our study population, some Bengals produced viable sperm as early as the second backcross generation, and Savannahs as early as the third backcross generation, although these are considered rare in the breeding communities, who regularly backcross early generation females to late generation, fertile hybrid males.

Fertility Evaluation

Determination of fertility was performed using one or more of three methods: Breeding records, semen evaluation, and

histopathology. Sterility was defined as repeated, confirmed matings with multiple proven females over 1 or more years with no conception, whereas fertile individuals were defined by documented breeding with validation via pedigree records. When available, electroejaculation was used to evaluate the presence of sperm and ultrastructural abnormalities (Howard et al. 1990). Cats with ejaculates possessing greater than 70% abnormal sperm (i.e., teratospermic) or exhibiting complete azoospermia were classified as sterile (Pukazhenthi et al. 2006), when breeding information was not available. Histopathological evaluation was performed on testes and epididymis from sexually mature males that underwent orchidectomy. Testes were laterally bisected and stored in Bouin's fixative. Testis and epididymis were embedded in paraffin, sectioned, and stained with H & E. Histology was evaluated to determine the presence/absence of germ cells, stage of meiotic progression, and the presence/absence of normal sperm. Fertile individuals possessed seminiferous tubules and caput epididymis with large numbers of sperm with normal morphology. Histological and semen evaluation data were available for all individuals utilized in RNA-Seq analyses.

Feline SNP Array

DNAs from 101 Bengals, 103 Savannahs, 20 African servals, and 13 Asian leopard cats were genotyped using the Illumina 63 k Feline SNP array, which assays 62,897 biallelic markers. Hybrid cats possessing a genotype call rate <0.90 were removed (99% of all study cats possessed call rates > 95%). Since the creation of the 63 K array, revisions to the feline genome required reassessment of the physical marker locations. The probe sequences were obtained and compared with FelCat5 using a local BLAST implementation (Altschul 1997) that identified 1,101 SNPs that could not be remapped to the genome assembly.

GWAS

Hybrid cats used for each binary case-control style GWAS were as follows: Bengals ($n = 60$ fertile; $n = 41$ sterile) and Savannahs ($n = 56$ fertile; $n = 47$ sterile) (supplementary fig. S1, Supplementary Material online). We searched for marker-based associations meeting or exceeding the Wellcome Trust recommendations ($P_{\text{uncorrected}} \leq 5 \times 10^{-5}$; $-\log_{10} P = 4.30$) (Wellcome Trust Case Control Consortium 2007). Notably, this significance threshold is conservative considering the polygenic nature of hybrid sterility and the modest SNP density of the Illumina feline SNP array, with the Wellcome Trust recommendations developed for a much higher density SNP array (Human Affymetrix 500 K GeneChip; see Wellcome Trust Case Control Consortium 2007). All marker-based association analyses were carried out using a mixed linear model, as described and implemented in EMMAX (Kang et al. 2010; Segura et al. 2012), and were executed in the SVS environment (Golden Helix, Version 7.7.6) as described (Neibergs et al. 2014). Briefly, the mixed model can be generally specified as: $y = X\beta + Zu + \epsilon$, where y is a $n \times 1$ vector of the observed phenotypes, X is a $n \times q$ matrix of fixed effects, β is a $q \times 1$ vector representing the coefficients of the fixed effects, and Z is a $n \times t$ matrix relating the instances of the random effect to the phenotypes of interest (Neibergs et al. 2014; http://doc.goldenhelix.com/SVS/8.2.1/mixed_models.html, last accessed June 10, 2015). Notably, we must assume that $\text{var}(u) = \sigma_g^2 K$ and $\text{var}(\epsilon) = \sigma_e^2 I$, such that $\text{var}(y) = \sigma_g^2 ZKZ' + \sigma_e^2 I$, but in this study, Z is simply the identity matrix I, and K is a kinship matrix among all samples. To solve the mixed model equation using the generalized least squares solution, the variance components (σ_g^2 and σ_e^2) must first be estimated as described previously (Kang et al. 2010; Segura et al. 2012; Neibergs et al. 2014; http://doc.goldenhelix.com/SVS/8.2.1/mixed_models.html, last accessed June 10, 2015). We used the REML-based EMMA approach to estimate the variance components (Kang et al. 2008), with stratification among hybrid cats accounted for and controlled using an IBS kinship matrix computed from the Illumina 63 K genotypes (Kang et al. 2010; Segura et al. 2012). Given precedence for dominance effects between the parental genomes of interspecific hybrids (Payseur and Hoekstra 2005; Chang and Noor 2007; White et al. 2012), we evaluated both dominant and additive models (fig. 2A, supplementary fig. S9, Supplementary Material online) using EMMAX, with subsequent comparison of the results. We also estimated the impact of including backcross generation as a covariate in our GWAS analyses and found that most of the top ranking markers described in this study remained, indicating that the kinship matrix corrects for most of the generation-based stratification. For example, in at least one model (i.e., Additive or Dominant with inclusion of backcross generation), markers SAV1–SAV3 ranked among the top 3 markers, whereas SAV5 and SAV4 were ranked among the top 6 or 7 markers, respectively. Likewise, BEN1 and BEN2 were ranked among the top 4 markers, whereas BEN3 (comprised two adjacent markers) ranked lower (i.e., among the top 31-32 markers). P-P plots are shown in supplementary figure S10, Supplementary Material online.

RNA-Seq and Expression Analysis

Whole-testis RNA was extracted from 25 mg of tissue using the Purelink RNA extraction kit (Life Technologies) for three fertile domestic cats, three fertile Bengals (assessed via histopathology and electroejaculation), three azoospermic (sterile) Bengals (two first and one third generation backcross hybrids), three fertile Savannahs (multiply proven breeders), and three azoospermic (sterile) Savannahs (one second- and two fourth-generation backcross hybrids). All animals were over the age of two and sexually mature. The integrity and concentration of total RNA samples was quantified using a Bioanalyzer 2100 system (Agilent). Fifty base-pair, single-end Illumina sequencing was executed for Bengal and domestic cat samples, and 100-bp single-end sequencing for Savannah, Asian leopard cat, and African serval samples using the Illumina HiSeq2000. RNAseq reads have been deposited under SRA accession numbers SRS913307–SRS913310, SRS913312, SRS913313, SRS913318, SRS913319, and SRS913323–SRS913330. Sequence reads were aligned to the

domestic cat genome (FelCat5) using a two-pass alignment strategy in the program *STAR* (Dobin et al. 2013). The alignments were sorted with *SAMtools* (Li et al. 2009). Polymerase chain reaction duplicates marked with *PicardTools* (http://broadinstitute.github.io/picard/, last accessed June 10, 2015) for the purpose of indel and SNP detection only. The GATK walker SplitNCigarReads was used to split reads into exons and hard-clip intronic sequences (McKenna et al. 2010). Mapping quality reassignment (MAPQ60) was applied to the alignments via GATK ReassignOneMappingQuality. Base quality score recalibration and indel realignment was performed on each individual separately, with SNP/indel discovery and genotyping across all 17 samples implemented simultaneously using variant quality score recalibration according to GATK Best Practices recommendations (Van der Auwera et al. 2013). Read count tables were generated using HTSeq-count (Liao et al. 2014). Reads were counted per-gene and as a union of all feature sets. Every feature with an *Ensembl* identifier was included in the differential expression set. Multiply mapped reads of equal quality were randomly assigned primary status and reads that mapped to more than five locations were discarded. We implemented several normalization approaches implemented in the EdgeR Bioconductor package (Robinson et al. 2010), including quartile normalization, trimmed mean of M (TMM) values, and relative log expression. To be conservative, we utilized the intersection of these approaches (supplementary fig. S5, Supplementary Material online) for downstream statistical analyses of chromosome expression bias and gene ontology enrichment. Relative expression differences between pairwise comparisons for the candidate genes were based on the TMM values. Chromosome-wide misexpression was tested for significance using a chi-squared test.

X Chromosome Duplication Analysis

We identified putative genomic duplications in the domestic cat version 6.2 assembly (felCat5) by remapping all Illumina fragment reads (Montague et al. 2014) and estimating copy number with CNVnator (Abyzov et al. 2011).

Supplementary Material

Supplementary figures S1–S10 and tables S1–S8 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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