Widespread formation of double-stranded RNAs in testis

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The testis transcriptome is highly complex and includes RNAs that potentially hybridize to form double-stranded RNA (dsRNA). We isolated dsRNA using the monoclonal J2 antibody and deep-sequenced the enriched samples from testes of juvenile Dicer1 knockout mice, age-matched controls, and adult animals. Comparison of our data set with recently published data from mouse liver revealed that the dsRNA transcriptome in testis is markedly different from liver: In testis, dsRNA-forming transcripts derive from mRNAs including promoters and immediate downstream regions, whereas in somatic cells they originate more often from introns and intergenic transcription. The genes that generate dsRNA are significantly expressed in isolated male germ cells with particular enrichment in pachytene spermatocytes. dsRNA formation is lower on the sex (X and Y) chromosomes. The dsRNA transcriptome is significantly less complex in juvenile mice as compared to adult controls, and possibly as a consequence, the knockout of Dicer1 has only a minor effect on the total number of transcript peaks associated with dsRNA. The comparison between dsRNA-associated genes in testis and liver with a reported set of genes that produce endogenous siRNAs reveals a significant overlap in testis but not in liver. Testis dsRNAs also significantly associate with natural antisense genes—again, this feature is not observed in liver. These findings point to a testis-specific mechanism involving natural antisense transcripts and the formation of dsRNAs that feed into the RNA interference pathway, possibly to mitigate the mutagenic impacts of recombination and transposon mobilization.

[Supplemental material is available for this article.]
(IFIH1, also known as MDA5), eukaryotic translation initiation factor 2 alpha kinase 2 (EIF2AK2, also known as PKR), adenosine deaminase RNA specific (ADAR), DExD/H-box helicase 58 (DDX58, also known as RIG-I) has been reported in response to various stressors that affect mitochondrial integrity or reduce transcriptional repression by DNA methylation (Hur 2019).

Because testis represents an immunologically privileged environment, the effects of dsRNA formation may be different in male germ cells as compared to somatic tissues (Zhao et al. 2014). Hence high levels of dsRNA formation as a result of pervasive transcription may be tolerated in male germ cells and have a specific purpose there. We have previously suggested an evolutionary mechanism involving antisense transcription, dsRNA formation, and endogenous siRNA (endo-siRNA) production to parse a viable sperm population from those that suffered catastrophic mutagenic events during recombination and transposon mobilization (Werner et al. 2009, 2015). The hypothesis is supported by the recent finding that the complex transcriptome in testis results in lower mutation rates in genes that are expressed during spermatogenesis (Xia et al. 2020).

Male germ cells display a highly complex transcriptome that potentially produces intermolecular dsRNA structures. Here, we enriched and sequenced dsRNA from mouse testis and compared it to a similar data set from somatic liver cells. We aimed to establish potential differences between the dsRNA transcriptome of male germ cells versus somatic (liver) cells. We then sought to establish whether dsRNA is a substrate for Dicer1 to generate endo-siRNAs (endo-siRNAs) and if natural antisense transcripts coexpressed with the cognate sense transcripts contribute form detectable levels of dsRNA.

Results
dsRNAs are enriched in mouse testis

Testes show the most complex transcriptome of all organs in mammals including a comprehensive array of natural antisense transcripts. We hypothesized that such transcriptional complexity may result in substantial formation of dsRNA that can be isolated using the dsRNA-specific antibody J2 (Dhir et al. 2018). Because RNA extraction methods introduce, depending on the particular methodology, either a significant positive or negative bias toward dsRNA, antibody pull-down of dsRNA was performed without prior RNA extraction. A hybridized sense–antisense transcript pair (slc34a2a/slc34a2as) from zebrafish (Nalbant et al. 1999) was added to the tissue lysate as a spike-in probe (Fig. 1A).

Testes of eight mice were used in the study: three juvenile (18 d old) wild-type (WT) mice, three age-matched male germ cell–specific Dicer1 knockout animals (Zimmermann et al. 2014), and two adult WT control mice. After incubation with the J2 antibody, both bound dsRNA and the flowthrough were sequenced. A total of 18.44 million reads were obtained from the antibody-bound samples (RIP) and 73.30 million reads from the flowthrough (FLOW); of these, 10.16 and 54.52 million reads, respectively, mapped uniquely to the mouse reference genome (Supplemental Table S1). The reads derived from the spike-in probe were almost entirely found in the RIP samples, although in some samples in very low numbers, indicating a comparably shallow read depth (Fig. 1C). Nevertheless, the significant enrichment of the dsRNA probe in the RIP samples confirmed the validity of the protocol and the specificity of the antibody.

Figure 2 includes two examples of aligned reads from a J2-enriched sample (R2) and a total RNA-seq testis experiment (Pervouchine et al. 2015), showing a cluster including the genes Gm37600, Bwr1, Ck1, Ppi3, Nif311, Orc2, Gmi15834, Fam126b, and Ndufb3 (194 kbp) that potentially form dsRNA on several occasions (Fig. 2A). The pattern is reflected in the total RNA-seq testis sample. The reads are not enriched in double-stranded regions, in line with the experimental procedure that recovers the entire transcripts that form hybrids and not only the complementary regions. An adjacent cluster (Aox4, Gmi15759, Aox2) with potentially overlapping transcripts is not expressed, hence no dsRNA was detectable. On the other hand, Kcnq1ot1 shows reads on...
Figure 2. Genome browser snapshots (SeqMonk) of representative examples of dsRNA-associated gene clusters. Two individual data sets with high read coverage from adult murine kidney (R2 and GSM900193) (Pervouchine et al. 2015) are shown. The upper panels represent the dsRNA-enriched sample (R2), the lower panels represent testis RNA (GSM900193). (A) The cluster encompassing the genes Bzw1, Clk1, Ppil3, Nif3l1, Orc2, Gm15834, Fam126b, and Ndufb3 shows reads mapping to both strands in exons of the related genes, although at low levels. There are hardly any regions that contain reads in both orientations. The adjacent cluster, also containing genes with complementary exons is not expressed. (B) Snapshot of the parentally imprinted Kcnq1 gene. The protein-coding sense transcript is not expressed in males; however, the related antisense transcript is expressed, and reads in both orientations are detected. This could be the result of intramolecular dsRNA formation by SINE and LINE elements enriched in this region. The blue bars represent the + strand and the red bars the – strand.
both strands with minimal expression of Kcnq1, possibly due to the clustering of LINE and SINE elements in Kcnq1ot1 that can form intramolecular dsRNA (Fig. 2B). Moreover, the read pattern is different between the J2-enriched and the total RNA-seq samples.

dsRNAs derive from testis- and liver-specific genic regions on autosomes

To characterize a comprehensive dsRNA transcriptome we pursued two strategies, one focusing on read peaks and the other on gene expression (Fig. 1B). The first one involved peak calling using genome coverage (genomecov, BEDTools) (Quinlan and Hall 2010), and calls from all samples were combined followed by the identification of genes associated with peaks. Combined reads from all eight samples were visualized per chromosome in relation to gene density (Fig. 3A). The dsRNA read depth follows the pattern of protein-coding gene density indicating that transcripts from these genes give rise to dsRNAs (Fig. 3A). Peaks that reached a threshold of five times more than background or higher were then annotated using ChIPpeakAnno (Zhu et al. 2010), and the genomic coordinates were compiled (Fig. 3B). Peaks were predominantly associated with protein-coding sequences, including regulatory features such as promoters, exons, and flanking regions confirming the matching appearance of read and gene densities in Figure 3A. The proportion between peaks in exons versus introns was 7.2 to 1. A total of 3328 peaks were detected on autosomes, 128 on the X, and 13 on the Y Chromosome, respectively, indicating a bias against the sex chromosomes. The vast majority of these peaks (97.2%) were associated with protein-coding and a few noncoding genes, whereas only 2.8% of peaks were found in intergenic regions. The sex chromosomes showed a comparable trend (97.6 vs. 2.4% on the X Chromosome and 84.6 vs. 15.4% on the Y Chromosome) (Fig. 3; Supplemental Table S2).

To investigate a potential difference between male germ cells and somatic cells, we used our pipeline to analyze a published data set from mouse fetal liver including J2-enriched samples and an input control (Gao et al. 2020). The experiments revealed a significantly different distribution of loci associated with dsRNA, particularly in peak numbers associated with exons versus introns. In testis the proportion was 37.3% to 5.2% compared to liver with 14.4% and 37.8% of total peaks, respectively (Fig. 3B). The overall difference between the two samples is highly significant ($\chi^2$, 89.92, $P$-value = 3.13 x $10^{-17}$). An additional difference between male germ cells and somatic cells concerns the 5′ flanking regions, which are associated with 4% of dsRNA peaks in testis but <1% in mouse liver, respectively. The sex chromosomes are distinct from autosomes in two features relevant to dsRNA formation, specifically repetitive
dsRNAs are highly abundant in pachytene spermatocytes

Because the majority of dsRNA-related read peaks in testis associate with annotated genes, we generated a list of the expressed transcripts using the RNA-seq quantitation pipeline in SeqMonk with a cutoff of eight times more than background and a “present” call in at least six of the eight samples. This approach produced a set of 3275 genes that form dsRNA in testis. Again, genes on the sex chromosomes are significantly underrepresented in this list (42 on the X Chromosome and one on the Y Chromosome vs. 3232 on autosomes) (Supplemental Table S2).

We used this list to obtain an impression of whether the genes associated with dsRNA formation show tissue-specific expression using publicly available transcriptome data (brain cortex, frontal lobe, cerebellum, lung, colon, spleen, kidney, bladder, heart, liver, and testis) (Pervouchine et al. 2015) as well as from staged developing male germ cells (premeiotic, pachytene, secondary spermatocytes, round spermatids) (da Cruz et al. 2016); round spermatids, elongated spermatids, and spermatozoa) (Zuo et al. 2016). Among the compilation of different tissues, testis expressed the highest proportion of dsRNA-forming genes with 27.6% versus 23.5–27.2% in other tissues (Fig. 4A). The percentage of expressed dsRNA-forming genes was elevated in isolated, developing male germ cells (33.3 ± 3.1%) and particularly in pachytene spermatocytes with a proportion of 58.1% ($P = $0.001) (Fig. 4B). Of note, the number of genes with positive calls is clearly reduced in pachytene spermatocytes and spermatozoa as compared to other stages. Despite the fact that the pipeline may only identify one transcript of a pair that forms dsRNA, the significant accumulation of mRNAs that form dsRNA (with an unidentified complementary transcript) in pachytene spermatocytes supports our focus on testis and suggests a biological role for dsRNA in this particular cell type.

We tested four loci with established complementary sense and antisense transcripts by RT-qPCR. Loci that contained a protein-coding gene and also produced a spliced, lowly expressed antisense transcript that shares complementarity in exons with the sense gene were selected. We designed four primer pairs for each sense–antisense pair that amplify fragments from noncomplementary and complementary regions of the mRNAs (Supplemental Fig. S2). Bidirectional transcription of the four loci was confirmed with a general trend that protein-coding sense transcripts are expressed at higher levels. Expression levels determined by RNA-seq and RT-qPCR did not correspond well, likely because RT-qPCR focuses on specific small regions, whereas RNA-seq quantification integrates the entire transcript.

The analysis of the dsRNA transcriptome has so far established that dsRNA is significantly more prevalent in testis, particularly in pachytene spermatocytes, than in somatic cells. Moreover, dsRNA in testis derives from annotated genic regions rather than intergenic sequences and introns.

Figure 4. Expression of potentially dsRNA-forming genes in various mouse tissues and staged male germ cells. (4A) Bar graph indicating the total number of expressed genes (100%, indicated above the bars) and the percentage of dsRNA-forming genes within the colored, lower part of the bars. Brown and red areas reflect the proportion of genes that form dsRNA in testis (dsRNA-associated genes), and the light gray areas are expressed genes without evidence of dsRNA. The different colors represent the data sets from Pervouchine (light brown), da Cruz (red), and Zuo (brown) (Pervouchine et al. 2015; da Cruz et al. 2016; Zuo et al. 2016). (4B) Compilation of all the values presented in A. Box plot indicating the median (solid black line) and 25th and 75th percentiles as box limits. Whiskers show 1.5 times the interquartile range; pachytene spermatocytes represent a clear outlier. $\chi^2$ test was performed, and related $P$-values are indicated.
dsRNAs are associated with endo-siRNAs and antisense transcripts

Significant levels of endogenous siRNA have been reported in testis, suggesting that the dsRNA may be processed by Dicer1 into endo-siRNAs (Song et al. 2011). To test this hypothesis, we first compared a published endo-siRNA data set from mouse testis (Hilz et al. 2017) with our dsRNA data. Moreover, we assessed the dsRNA transcriptome from juvenile, male, germ cell–specific Dicer1 knockout (KO) mice and age-matched wild-type littermate controls on the assumption that Dicer1 may be involved in dsRNA processing. Reads were aligned and expression quantified using the pipeline for peak calling as applied previously for the testis and liver data sets. To determine overlapping regions, the coordinates of genes associated with dsRNA and with endo-siRNA were intersected using the online tool BedSect (https://imgsb.org/bedsect/) (Mishra et al. 2020). We compared the dsRNA-associated genes from mouse testis (3492 entries) and liver (RIP, 4900 entries, and input control, 4061) to the published data set of endo-siRNAs (3712 entries) in testis (Hilz et al. 2017) as well as to a list of annotated natural antisense genes (2991 entries, Ensembl BioMart).

The coordinates of 1000 randomly selected dsRNA genes from mouse testis and liver were intersected with endo-siRNA-associated genes. The number of genes with dsRNA and endo-siRNA formation was comparable in mouse liver J2-enriched samples and input control (401.2 ± 15.2 vs. 401.4 ± 11.4 per 1000 genes) but significantly different from a set of genes associated with dsRNA reads in testis (468.3 ± 11.2; P < 0.0001) (Fig. 5A). This result was confirmed by comparing normalized reads in mouse testis RIP and FLOW samples. The RIP samples displayed about 10 times as many normalized reads that intersected with siRNA signals as counted with the FLOW samples (140.04 vs. 15.37 RPKM, P < 0.0001, respectively) (Fig. 5B). Three chromosomes showed higher counts in the FLOW samples (Chr 1, 3, and 10) caused by highly expressed peaks that skew the otherwise “normal” proportion (Supplemental Fig. S3). Of note, X and Y Chromosomes display a significantly lower number of dsRNA reads that correlate with endo-siRNAs (Fig. 5B).

We then interrogated the two dsRNA-enriched samples from mouse testis and liver (same data sets as for the endo-siRNA analysis) for a potential association with natural antisense transcripts. The coordinates of antisense genes (2991) were intersected with 1000 randomly selected dsRNA-associated genes from mouse testis and liver (3492 and 4900 genes, respectively). Again, the input sample from mouse liver was used as a control. As shown in Figure 5C, both dsRNA-enriched samples (testis and liver) are associated with antisense transcripts, although in testis the link is clearly more pronounced (input control 148.3 ± 12.3 vs. 168.9 ± 10.2 and 237.8 ± 11.4, liver and testis, respectively). Moreover, the association between dsRNA and antisense transcripts was significantly higher in the J2-enriched as compared to the FLOW samples (P < 0.0001) (Fig. 5D).

The second strategy to investigate a potential link between dsRNA and endo-siRNA focused on the dsRNA transcriptome from juvenile, male germ cell–specific Dicer1 knockout (KO) mice and age-matched wild-type littermate controls. The late phases of spermatogenesis are severely disrupted in Dicer1 KO mice (Korhonen et al. 2011); therefore, we used testes from 18-d-old juvenile mice before the onset of spermatogenic defects.

In general, the dsRNA transcriptome was comparable between juvenile WT and Dicer1 KO mice (316 vs. 364 peaks) but significantly less complex than in adult WT mice (3461 peaks). Again, the peaks are predominantly associated with protein-coding genes, as noted previously in testis (Song et al. 2011). However, the peak density is increased in juvenile WT mice compared to adult WT and juvenile KO mice (Supplemental Fig. S4).

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sequences, with <10% mapping to introns or intergenic regions (Fig. 6A). However, almost half of the genes (148) were differentially expressed: 95 were significantly overexpressed (P ≤ 0.05), and 53 were suppressed in Dicer1 KO animals (Supplemental Table S3). Of note, none of the 148 genes mapped to the mitochondrial genome, which produces the highest density of dsRNA. The peak finding pipeline applied to the WT and Dicer1 KO samples failed to establish significant differences in dsRNA peak occurrence (Fig. 6A). We next overlapped the genomic regions of J2-bound peaks in the three data sets (juvenile WT, Dicer1 KO, and adult WT mice). We found 144 genes to be present in all three data sets and only 44 and 17 peak-associated genes solely present in WT mice. We found 144 genes to be present in all three data sets (juvenile WT, Dicer1 KO, and WT samples with the endo-siRNA and antisense gene coordinates and compared it to size-matched control sets of random dsRNA-associated genes. Figure 6, E and F, indicate that the overlap between dsRNA genes and siRNA-related genes is comparable between adult and juvenile animals, whereas the juvenile data sets contain clearly less antisense-associated peaks (P < 0.001). Of note, these experiments involved juvenile mice, and the complexity of the dsRNA transcriptome is expected to increase with age as may the contribution of Dicer1 in processing dsRNA (Björkgren and Sipilä 2015).

**dsRNA formation is essential for male germ cell development**

Finally, we generated a parsed list of dsRNA genes by intersecting the two dsRNA lists from testis (1893) (Fig. 1B; Supplemental Table S2). We used this list of dsRNA-related genes to assess phenotypic consequences of a single gene knockout (Fig. 7). The same approach was performed with the dsRNA-associated genes in liver as a comparison. We also examined the extent to which dsRNA-forming genes contributed to specific phenotypes on the background of genes expressed in testis (Pervouchine et al. 2015) using Genomic Regions Enrichment of Annotations Tool (GREAT) analysis (McLean et al. 2010).

In general, the knockout of dsRNA genes had a strong influence on cellular and embryonic development related to the investigated tissue, that is, sperm development in testis (Fig. 7A,B; Supplemental Table S4). The knockout of genes present in both data sets (1759) was predominantly associated with defects in embryonic development (Supplemental Fig. S4). The same approach with the liver input control, again, showed an enrichment of developmental phenotypes, reflecting a tightly regulated transcripational program rather than a particular role of dsRNA formation (Supplemental Table S4). When the dsRNA-generating genes in testis were assessed for enrichment on a testis transcriptome background, traits related to sperm morphology, male germ cell apoptosis, and developmental arrest constituted the first 12 entries of a list of 14 terms that showed significant enrichment (Fig. 7C). These findings suggest that dsRNA formation may constitute an essential checkpoint for male germ cell development.

We made a considerable effort to visualize dsRNA in adult mouse tissue by fluorescence immunohistochemistry using the

![Figure 6](image-url)
J2 antibody. However, the signal did not reach the detection limit. As a positive control, we used cultured cell lines (A375 and CCD1106) and treated the cells either with the dsRNA analog poly I:C or stressed them with azacytidine to provoke endogenous dsRNA production. Both procedures resulted in clearly enhanced staining with the J2 antibody, confirming on the one hand the specificity of the antibody and on the other suggesting that the level of endogenous dsRNA is low and spatially dispersed (Supplemental Fig. S5). An additional limitation of this study is the different mouse strains used for J2 enrichment of dsRNA. The processing of dsRNA and its role in innate immunity are well-established and generally conserved in vertebrates. Therefore, it is unlikely that

Figure 7. Consequences of dsRNA-associated gene deletion on mouse phenotypes using GREAT (McLean et al. 2010). Only the Gene Ontology “Mouse Phenotype Single knockout” is shown. The list of the top 20 enriched terms of the other gene ontologies is given in Supplemental Table S4. (A) The parsed list of 1893 genes that form dsRNA was tested for enrichment against all protein-coding mouse genes (21,395). The mouse phenotype single KO database contains 9170 entries that cover 9466 or 44% of all genes. (B) Phenotypes enriched after knockout of dsRNA genes expressed in mouse liver on the background of all genes. (C) Phenotypes enriched after knockout of dsRNA genes expressed in mouse testis (1888 genes or 16%) on a background of genes that are expressed in testis (11,649).
strain-specific differences account for the reported differences between the analyzed data sets.

To conclude, we have shown that the dsRNA transcriptome in testis is fundamentally different from that in liver. Moreover, our evidence suggests that the dsRNA structures are formed between natural sense–antisense transcripts, recognized by DICER1, and processed into endo-siRNAs. These findings corroborate a testis-specific biological role of dsRNA for which the dsRNA structure of the molecule is the key determinant for function rather than the protein-coding potential of the particular genes.

Discussion

The highly complex transcriptome of testis appears to greatly exceed the demands of functioning simply as a (reproductive) organ (Soumillon et al. 2013). Various explanations have been offered, ranging from transcriptional fallout after DNA demethylation to RNA- or transcription-dependent genomic quality control (Werner et al. 2015; Xia et al. 2020). The recent findings that genes transcribed during sperm development show lower mutation rates than silent loci strongly support the latter hypothesis and emphasize the role of transcription and transcription-related repair (Xia et al. 2020). However, no mechanistic insights to underpin such biological role have been reported so far. Our investigations here suggest a mechanism during sperm development that involves dsRNA formation from genic regions including natural antisense transcripts followed by processing into endo-siRNAs. Failure of the mechanism appears to interfere with sperm development and promote apoptosis of male germ cells.

Analysis of the dsRNA transcriptome of fetal mouse testis and liver indicates that mitochondria contribute substantially to the dsRNA transcriptome (Dhir et al. 2018; Kim et al. 2018); conversely, nuclear transcripts that form dsRNA show significant differences regarding their origin (Fig. 3B). In testis, >90% of dsRNA-forming transcripts are associated with mRNA features, including promoters as compared to 58% in liver. The difference suggests that fully processed mRNAs significantly contribute to the formation of dsRNA in testis. Our analysis indicated that dsRNA formation in testis occurs in pachytene spermatocytes, whereas the cellular origin of dsRNA in liver is less clear. The observation that the knockout of dsRNA-forming genes in liver is associated with developmental defects indicates that dsRNA formation in undifferentiated cells may play a biological function. Accordingly, stem cells tolerate dsRNA in the cytoplasm without triggering an immune response (Wang et al. 2013).

A recent study has compared single-cell sequencing from mouse and human male germ cells and found comparably high levels of genic transcription in both species, predominantly in spermatocytes and round spermatids (Xia et al. 2020). Of note, these are also the stages identified in this study that express the highest proportion of dsRNA-forming genes. The particular and prominent expression of dsRNA in male germ cells raises the question whether oocytes show a comparable expression pattern. Studies using genetically modified mice with mutations in retrotransposon defense mechanisms have identified three specific pathways that protect the oocyte genome, including piRNAs, RNA interference, and transcriptional silencing mechanisms controlling LINE-1 elements (Taborska et al. 2019). The dsRNA feeding into piRNA and endo-siRNA pathways in oocytes derives from repetitive elements; whether genetic dsRNA formation as observed in male germ cells occurs also in oocytes remains to be established.

The formation of endogenous dsRNA comes with a significant risk for mammalian cells. RNA duplexes of 30 bp and longer are reminiscent of dsRNA viruses and recognized by sensor proteins that trigger a strong innate immune response (Wang and Carmichael 2004). The discoveries of RNA interference and the widespread antisense transcription in mammalian genomes have indicated that, despite the danger of eliciting an unwanted immune reaction, endogenous dsRNA formation occurs (Carlile et al. 2008; Ghildiyal et al. 2008; Okamura and Lai 2008; Watanabe et al. 2008). However, the nature of the dsRNA transcriptome established here suggests that RNA duplexes have distinctly different biological roles in sperm and somatic cells. In the latter, dsRNA is contained in mitochondria and the nucleus and only leaks out when the barriers break down or production is increased owing to pathologies or drugs (Tarallo et al. 2012; Tsai et al. 2012). Accordingly, the inactivation of polynucleotide phosphorylase (PNPase), which breaks down mitochondrial dsRNA, leads to IFIH1 activation and ultimately triggers an interferon-mediated innate immune response (Dhir et al. 2018). Moreover, drugs that reduce DNA methylation and increase spurious transcription of nuclear Alu elements (e.g., azacytidine) induce a dsRNA response (Ahmad et al. 2018). The transcripts generated from repetitive elements are generally not processed and remain in the nucleus and are thus segregated from the dsRNA sensors in the cytoplasm (Kiyosawa et al. 2005; Elbarbary et al. 2016).

The situation in testis presents differently. Here, transcripts that form dsRNA derive from genic regions and are spliced, hence they are more likely to reach the cytoplasm. The mouse gene expression database (http://www.informatics.jax.org/expression.shtml) and the Human Protein Atlas (https://www.proteinatlas.org/) indicate that IFIH1 and EIF2AK2 (also known as PKR) are expressed at a low to medium level in developing male germ cells, whereas DICER1 and ADAR in the nucleus are more abundant. Accordingly, spermatocytes show a reduced response to poly I:C, a synthetic dsRNA analog widely used to experimentally trigger an antiviral response (Li et al. 2012). Male germ cells may therefore constitute a cellular environment that is more tolerant against cytoplasmic dsRNA essential for a posttranscriptional regulatory role of dsRNA.

The connection between dsRNA and a processing by DICER1 into endo-siRNAs is well-established in Caenorhabditis elegans (Duchaine et al. 2006; Vasale et al. 2010) and Drosophila (Czech et al. 2008; Ghildiyal et al. 2008; Lucchetta et al. 2009), less so in vertebrates (Watanabe et al. 2006, 2008; Carlile et al. 2009). Nevertheless, endo-siRNAs in mouse testis have been characterized previously, and a regulatory role reminiscent of miRNA function has been proposed (Song et al. 2011). Limited endo-siRNAs have also been found in a human cell line (HEK293), potentially linked to sense/antisense transcript pairs, but with unknown cellular function (Werner et al. 2014).

Our results confirm the link between dsRNA and the formation of endo-siRNAs, although the depletion of DICER1 in the testes of young mice only marginally affected the levels of dsRNA, which would be expected if DICER1 is efficiently processing RNA hybrids. The key concern relates to the young age of the Dicer1 KO animals and the limited complexity of the dsRNA transcriptome at this stage, which makes a potential impact of the knockout difficult to monitor. On the other hand, the minor bias toward overexpression of a heterogeneous (small) group of genes in KO animals is consistent with a role for DICER1 in processing dsRNA. Of note, DICER1 is localized to the chromatoid body in spermatocytes and round spermatids where also most of the
polyadenylated RNA is accumulated (Kotaja et al. 2006; Jiang et al. 2020). A related observation was also made by Zimmermann and coworkers who reported a small up-regulation of protein-coding genes in Dicer1 KO animals (Zimmermann et al. 2014). Of importance, however, DICER1 is essential for miRNA processing and a general stimulation could also be the effect of decreased levels of miRNAs (Korhonen et al. 2011).

A recurrent and striking feature in the analysis of dsRNA is the distinct underrepresentation of dsRNA peaks, dsRNA-associated genes, as well as endo-siRNAs mapping to the X and Y Chromosomes. A similar bias against the X Chromosome has also been observed in the context of antisense transcripts generated from the mouse (and human) genome (Kiyosawa et al. 2003; Chen et al. 2004). The X Chromosome bias is not observed in sense-antisense transcript pairs with complementarity restricted to introns. Our findings that introns contribute only marginally to dsRNA structures in testis (Fig. 3B) and that dsRNA is associated with antisense transcription (Fig. 6C) are in line with the early observations by Kiyosawa et al. (2003) and Chen et al. (2004). Accordingly, we could corroborate the link between natural antisense transcripts and dsRNA formation in testis with a less pronounced association between dsRNA-associated genes and antisense transcripts in liver (Fig. 6). This observation contrasts with the accumulation of repetitive elements on the X Chromosome (Komissarov et al. 2011). The significant contribution of Alu and LINE-1 elements to dsRNA formation in somatic cells (Sadeg et al. 2021) may again point to different biological roles of dsRNA formation in germ and somatic cells.

The analysis of mouse phenotypes with deletions of dsRNA-associated genes suggests an essential role of these genes and potentially dsRNA formation in developmental processes. In tests, knockouts affected disproportionately sperm morphology and caused male germ cell apoptosis. These observations concur with a model in which genic transcription followed by dsRNA formation and siRNA production enables a control mechanism to mitigate DNA damage from recombination and transposon mobilization (Werner et al. 2015).

Visual scrutiny of dsRNA-related peaks indicated that very often the genes were indeed transcribed in both directions, but the reads did not necessarily map to the complementary parts of the transcripts (Fig. 2). This was also observed with the spike-in reads that mapped to single-stranded rather than complementary regions, suggesting a bias against sequencing double-stranded structures. The sequencing bias against RNA hybrids makes it impossible to unambiguously match the interacting sense-antisense transcripts. This also highlights a major experimental challenge when investigating dsRNA structures. Experimental strategies and procedures such as RNA extraction and reverse transcription are generally optimized for single-stranded molecules, and dsRNA may show a different behavior. For example, guanidinium salts as used in TRIzol strongly promotes double-strand formation (Mölder and Speek 2016), whereas standard reverse transcription and library synthesis as used here are inhibited by long dsRNA stretches. These considerations suggest that occurrence of dsRNA-forming sense-antisense transcript pairs may be underestimated here because only one part of the complementary gene pair was used for the intersections.

To conclude, we have shown that the dsRNA transcriptome in testis is fundamentally different from somatic liver cells. Moreover, our evidence suggests that the dsRNA structures involve pairs of natural sense-antisense transcripts. The RNA hybrids are processed into endo-siRNAs, most likely by DICER1. These findings are in line with the highly complex transcriptome and suggest a testis-specific biological role of dsRNA in which the double-strand structure is the key determinant rather than the protein-coding potential of the particular genes.

Methods

Animals

The mice used in this study were three Dicer1 KO mice (18 d old) (for details, see Korhonen et al. 2011), three age-matched wild-type juvenile mice (18 d old), and two adult control mice (BALB/c, ca. 4-6 mo old). Mice were housed under a controlled environment (12 h light cycle, temperature 22°C, humidity 55%±15%, specific pathogen free) at the Central Animal Laboratory of the University of Turku. Standard pellet chow and reverse osmosis water were available ad libitum. Male germ cell–specific Dicer1 knockout mice were generated as previously described by crossing mice with floxed Dicer1 alleles with mice expressing transgenic Cre under the neurogenin 3 (Neurog3) promoter (Korhonen et al. 2011). Dicer1 (fx/wt) littermates without Cre expression were used as controls. The mice were of mixed genetic background (C57BL/6J and C3H129). All procedures were performed in accordance with Finnish laws and the Guide for Care and Use of Laboratory Animals (National Academy of Science, License number: 2009-1206-Kotaja).

Spike-in probe

Plasmids encoding the natural sense-antisense transcript pair (slc34a2a and slc34a2as from zebrafish) (Nalbant et al. 1999) were linearized with Xbal and transcribed in vitro using the MEGAscript T7 Transcription Kit (Invitrogen). The transcripts are 2607 bases (sense, NM_131624) and 1371 bases (antisense, NR_002876.2) long and share 563 bp of complementarity over two exons. The resulting RNA was quantified and mixed in equimolar concentrations to a total concentration of 0.4 µg/µL. One microliter was diluted 500× with 0.1 M NaCl, heated to 70°C, and gradually cooled to hybridize the two strands. One microliter of the spike-in probe was added (0.8 ng) to the testis homogenate before J2 binding (see below).

Double-stranded RNA immunopurification

The protocol published by Dhir and coworkers was followed with slight modifications (Dhir et al. 2018). Both testes of the juvenile mice and half of a testis of adult mice were homogenized in 220 µL of NP-40 lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% Na-deoxycholine, 220 units RNasin) using a disposable pestle followed by DNA shearing with a 25G needle. Cell debris was removed by centrifugation, and the volume was increased to 1 mL per sample with NET2+DOC buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM MgCl2, 0.5% Na-deoxycholine, 0.2 units/µL DNase l) plus 5 µg of J2 antibody (Scicons 10010500). At this point, the spike-in probe was added. Samples were rotated for 3 h at 4°C, then 100 µL of μMACS Protein G MicroBeads (Miltenyi Biotec) per sample was added followed by a 1-h incubation. The samples were then loaded onto μMACS columns equilibrated with NP-40 buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40), and the flowthrough (FLOW) was collected. Columns were washed with 300 µL of NP-40 and 3× 250 µL of wash buffer (50 mM Tris pH 7.5, 1 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% Na-deoxycholine, and 0.1% SDS). The columns were removed, and beads with dsRNA were washed off the column with water. Beads and dsRNA were mixed with TRIzol and purified.
according to established protocols. In parallel, the RNA from 200 µL of the FLOW were purified with TRIzol and used as a background.

Sequencing

RNA samples were quantified, and the integrity was tested using a Bioanalyzer (Agilent). Strand-specific RNA-seq libraries were prepared using the NuGEN Ovation SoLo kit (Ovation SoLo RNA-seq System, Human, Teycan) without size selection or fragmentation of RNA. The supplier’s guidelines were closely followed with the exception that the antibody-bound samples were excluded from rRNA depletion, whereas the flowthrough samples were rRNA depleted. The stranded library contained inserts of 300–350 bp. Paired-end sequencing was performed on an Illumina HiSeq 2500 platform at the Kinghorn Centre for Clinical Genomics, Garvan Institute of Medical Research (Darlinghurst, Australia) (sequencing read length 42–117 bases).

Data analysis

The quality of reads was assessed using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and adapters were trimmed using Trimmmomatic (version was 0.3.6) (Bolger et al. 2014). The spike probe reads in the different samples were aligned to a fragment of zebrafish Chromosome 1 (Chr 1: 14,432,434–14,454,662) that contains the slc34a2a gene and the related natural antisense transcript (slc34a2aas) originating from the bidirectional Rbpia promoter using STAR version 2.5.2b (Dobin et al. 2013). All data sets were then quantified using Salmon (Patro et al. 2017), and expression differences between KO mice, juvenile wild-type mice, and adult controls were established using DESeq2 (Love et al. 2014). All data sets were then quantified using DESeq2 (Love et al. 2014).

The list for dsRNA-forming genes was then intersected with the expressed genes in different tissues and male germ cells that were aligned and quantitated using the STAR/SeqMonk pipeline. Then, dsRNA-derived reads were assessed in two different ways, with default settings. In the first, dsRNA-forming reads were used to establish the genome coverage and distribution for the individual samples. Flowcharts summarizing the different pipelines are provided in Supplemental Figure S1. Murine tissue expression data from Pervouchine et al. (2015) was accessed from the NCBI Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE63025 (deposited under NCBI BioProject [https://www.ncbi.nlm.nih.gov/bioproject/] study PRJNA66167). Sequencing data from isolated, staged male germ cells were accessed from the NCBI BioProject study PRJNA317251 (da Cruz et al. 2016) and the NCBI Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra) under accession number SRP078798 (Zuo et al. 2016). Short RNA reads from mouse testis were accessed from GEO accession number GSE83264 (Hilz et al. 2017). Gene Ontology and Mouse Phenotype Single KO analysis were performed using GREAT (McLean et al. 2010). A minimal enrichment of twofold was set with a cutoff of 20 terms. As background for enrichment the 21,395 protein-coding mouse genes (NCBI build 38; UCSC mm10, December 2011) were used for testis and liver samples. Alternatively, the testis sample was also tested for enrichment against the protein-coding genes expressed in testis as determined using the SeqMonk pipeline (11,622). False discovery rates as well as P-values were determined.

RT-qPCR

For each gene, four primer pairs were designed to analyze the expression of (1) the sense gene with at least one exon overlap with the antisense gene, (2) the sense gene with no exon overlap with the antisense gene, (3) the antisense gene with at least one exon overlap with the sense gene, and (4) the antisense gene with no exon overlap with the sense gene (Supplemental Fig. S2; Supplemental Table S5). Actin, beta (Actb) was used as a reference gene. First, a DNA digestion step was implemented (DNase I, Promega) followed by SigmaSpin Sequencing Reaction Clean-Up (Sigma-Aldrich). RT-qPCR was performed using the Luna Universal One-Step RT-qPCR Kit (NEB) according to the manufacturer’s instructions, in a LightCycler 480 System (Roche) with the following parameters: Reverse transcription for 10 min at 55°C; initial denaturation for 1 min at 95°C; denaturation for 10 sec at 95°C and an extension for 30 sec at 60°C for 45 cycles; melting curve at 95°C. The RNA used was from RT-qPCR was from an 18-d-old mouse testis sample.

Immunofluorescence

For immunohistochemistry, 5-µm sections of adult mouse testis fixed in 4% paraformaldehyde/0.1% Triton X-100 in 5% normal serum in TBS (30 min). Unspecific binding was blocked with 5% bovine serum in TBS (30 min). The primary J2 antibody (Scicins 10010500) was diluted 1:200 (1 µg/µL stock), the secondary antibody, Alexa Fluor 488 (A-21131) or Alexa Fluor 594-coupled goat anti-mouse IgG2a (Thermo Fisher Scientific A-11032), was used at 1:1000. The cell nuclei were counterstained using DAPI (4′,6-diamidino-2-phenylindole). Sections were washed with TBS, mounted using VECTASHELD (Vector Laboratories) and imaged using a Unit Zeiss AxioImager1 fluorescence microscope.

Immunohistochemistry was performed to test the specificity of the J2 antibody and to examine the expression of dsRNA-binding proteins. CCD1106 keratinocytes or A375 cells were treated with poly I:C (0.5 µg/mL) or acetylidine (500 nM) for 24 h. Cells were washed with PBS and fixed with 4% paraformaldehyde in PBS (Affymetrix) for 10 min. Cells were permeabilized in 0.25% Triton X-100 followed by blocking with 3% BSA in PBS.
Antrim fraction V, USB) for 1 h. The antibodies used were J2 (Sicons 10010500, 1:200), PKR (Abcam ab32052, 1:1000), IFH1 (Abcam ab126630, 1:1000) and RIG-I (Abcam EPR186629, 1:1000), incubation was for 1 h at room temperature. Cells were washed with PBS-Tween and then incubated with the secondary antibodies Alexa Fluor 488 goat anti-mouse IgG2a (Thermo Fisher Scientific A-21131) or Alexa Fluor 594 goat anti-rabbit IgG H+L (Thermo Fisher Scientific A-11032) and analyzed as above.

Data access
Raw and processed sequencing data generated in this study have been submitted to the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/) under accession number PRJA630221. Codes generated for this work are available as Supplemental Code and at GitHub (https://github.com/James-E-Clark/Masters-dsRNA-Project and https://github.com/jwcaseament/dsRNA-seq-project).

Competing interest statement
The authors declare no competing interests.

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