Supplementary Note

Fertility Defects in *pi6* Mutant Male Mice Are Not Due to Abnormal Litter Size or Mating Behavior

The reduced progeny from *pi6*$^{em1/em1}$ males does not reflect fewer pups produced in each litter: *pi6*$^{em1/em1}$ males sired 5 ± 2 (n = 4) pups per litter compared to 6 ± 2 (n = 27) for C57BL/6 control males (Fig. 2a). Moreover, *pi6*$^{em1/em1}$ males regularly produced mating plugs, a sign of coitus, in cohabiting females. The observed fertility defects are specific for the loss of *pi6* piRNAs in males because *pi6*$^{+/em1}$ heterozygous males and *pi6*$^{em1/em1}$ homozygous mutant females showed no discernable phenotype.

pi6 Is Not Required in the Soma of the Developing Embryo

pi6 piRNAs appear to play little if any role in the soma of the developing embryo. *pi6*$^{+/em1}$ males mated to *pi6*$^{+/em1}$ females yielded progeny at the expected Mendelian and sex ratios. Moreover, the weight of *pi6*$^{em1/em1}$ homozygous pups (28.3 ± 0.6 g, n = 6) that developed to adulthood was indistinguishable from their C57BL/6 (26.9 ± 0.3 g, n = 6) or heterozygous littermates (28.6 ± 0.3 g, n = 8; Extended Data Fig. 2b). We detected no difference in the gross appearance or behavior among these pups.

The Majority of *pi6* Mutant Sperm Have Normal Morphology

Two-to-four months after birth, both *pi6*$^{+/em1}$ and *pi6*$^{em1/em1}$ testes weighed ~15% less than wild-type C57BL/6 testes (Extended Data Fig. 2b). Nonetheless, *pi6*$^{em1/em1}$ and *pi6*$^{em2/em2}$ testes had normal gross histology, with all expected germ cell types present in seminiferous tubules and sperm clearly visible in the lumen (Fig. 2d). Despite the normal quantity of sperm, *pi6*$^{em1/em1}$ sperm showed signs of agglutination after 90 min incubation in vitro, compared to C57BL/6 sperm. Moreover, 11 ± 3% (n = 4) of *pi6*$^{em1/em1}$ caudal epididymal sperm had abnormal head morphology (Extended Data Fig. 2c),

compared to 2 ± 1% (n = 5) of wild-type sperm (p = *0.02*). Defects in germ cell chromosomal synapsis, triggering errors in gene expression, have been linked to abnormal sperm head shape[1,2]. In fact, 22 ± 7 percent (n = 4) of *pi6*$^{em1/em1}$ pachytene spermatocytes had unsynapsed sex chromosomes or incompletely synapsed autosomal chromosomes, compared to 7 ± 3 percent (n = 4) for C57BL/6 (Extended Data Fig. 2d and 2e).

pi6 Mutants Continue to Repress Transposons

We used RNA-seq to measure the abundance of RNA from 1,007 transposons in four distinct purified germ cell types: pachytene spermatocytes (4C), diplotene spermatocytes (4C), secondary spermatocytes (2C), and spermatids (1C). *pi6* piRNAs compose 5.5% of all spermatocyte pachytene piRNAs (943,758 molecules per diplotene spermatocyte; Extended Data Fig. 3b), yet when *pi6* piRNAs were eliminated in *pi6*$^{em1/em1}$ or *pi6*$^{em2/em2}$ mice, we found no significant changes in steady-state RNA abundance (i.e., an increase or decrease >2-fold and FDR <0.05) for any transposon family compared to C57BL/6 germ cells (Extended Data Fig. 3c). We also note that, as in C57BL/6 wild-type, ɣH2AX expression was confined to the sex body in pachytene spermatocytes in *pi6*$^{em1/em1}$ testis, indicating an absence of DNA damage (data not shown). Together with the rescue of the fertilization defects of *pi6*$^{em1/em1}$ sperm by ICSI, these data suggest that transposon silencing is unlikely to be the essential biological function of *pi6* piRNAs.

Impaired Motility in *pi6* Mutant Sperm

Only 10 min after sperm extraction, most *pi6*$^{em1/em1}$ sperm moved more slowly than C57BL/6 control sperm (Supplementary Movies 1 and 2), and as the sperm were incubated in capacitating conditions, motility of the mutant sperm continued to declined more rapidly than that of control (Supplementary Movies 3–10). By 4 h, most *pi6*$^{em1/em1}$

sperm only moved in place and showed signs of agglutination (Supplementary Movies 8 and 10).

IVF- and ICSI-derived *pi6* Heterozygous Embryos Suffer Impaired Preimplantation Development

The low number of fertilized two-cell embryos produced in IVF using *pi6*$^{em1/em1}$ sperm precluded transferring the standard number of embryos to surrogate mothers. For example, in two IVF experiments using *pi6*$^{em1/em1}$ sperm, only 5 or 7 embryos could be transferred; the surrogate females failed to become pregnant (Fig. 5c and Extended Data Fig. 4a, Trials 1 and 2). In theory, this result might suggest a paternal role for *pi6*. A more mundane explanation is that the low number of embryos transferred reduced the yield of live fetuses, as reported previously[3–5]. We conducted additional experiments to distinguish between these two possibilities. Oocytes were again fertilized by IVF with *pi6*$^{em1/em1}$ or C57BL/6 control sperm, and two-cell embryos transferred to surrogate females, but matching the number of embryos transferred to each surrogate for the two sperm genotypes. We used two strategies. First, similar numbers of embryos derived from *pi6*$^{em1/em1}$ sperm and wild-type "filler" embryos derived from control sperm were transferred to separate oviducts in the same females to make up the total numbers of transferred embryos (Fig. 5c, Trials 3 and 4). Again, fewer embryos developed to term for *pi6*$^{em1/em1}$ (25% for Trial 3 and 4) compared to control sperm (66% for Trial 3 and 4). Second, embryos derived from *pi6*$^{em1/em1}$ sperm and wild-type filler embryos were mixed before transfer and then equal numbers of embryos, selected randomly, were implanted in each oviduct (Fig. 5c, Trial 5). Pups isolated by cesarean section 18.5 days after transfer were genotyped by PCR. In this experiment, only 40% of embryos derived from *pi6*$^{em1/em1}$ sperm developed to term, compared to 80% of wild-type filler embryos. Finally, in one experiment (Fig. 5c, Trial 6) where we obtained sufficient numbers of embryos derived from *pi6*$^{em1/em1}$ sperm, 10 *pi6*$^{em1/em1}$-derived two-cell embryos were

transferred to each oviduct of the surrogate female. Just 15% of the *pi6*$^{em1/em1}$-derived embryos developed to term, compared to 85% for C57BL/6. Together, these data suggest a paternal role for *pi6* piRNAs in the embryo.

Further support for the idea that paternal *pi6* piRNAs play a role in embryogenesis or embryonic viability comes from transfer of embryos generated by ICSI (Fig. 5d). ICSI with *pi6*$^{em1/em1}$ or *pi6*$^{+/em1}$ sperm yielded comparable normal numbers of fertilized oocytes (Fig. 3b), so no wild-type filler embryos were used; all embryos were transferred into a single oviduct of the surrogate female. In two independent experiments in which embryos generated by ICSI were transferred to surrogate mothers, only 19% of two-cell embryos derived from *pi6*$^{em1/em1}$ sperm heads developed to term, compared to 34% for embryos fertilized with *pi6*$^{+/em1}$ (Fig. 5d). Only four of seven (57%) surrogate mothers carrying embryos derived from *pi6*$^{em1/em1}$ sperm became pregnant. All three surrogate mothers receiving embryos derived from *pi6*$^{+/em1}$ sperm became pregnant (Extended Data Fig. 4b).

We note that the live fetuses generated using *pi6*$^{em1/em1}$ sperm in IVF or sperm heads in ICSI, like those produced by natural mating using *pi6*$^{em1/em1}$ males, showed no obvious morphological abnormalities and grew to adulthood normally when fostered by host mothers. Our data suggest a role for paternal *pi6* piRNAs in early embryogenesis or embryo viability.

pi6 Promoter Deletion Does Not Cause Large-Scale Changes in the Expression of Neighboring Genes

In theory, disruption of *pi6* could influence flanking gene expression, confounding transcriptome analysis. However, we find no evidence for coincidental changes in the expression of the genes flanking *pi6*. In *pi6* mutant pachytene spermatocytes, diplotene spermatocytes, and secondary spermatocytes, no gene on chromosome 6 is affected except for *pi6* itself. In spermatids, the steady-state mRNA abundance of *Atp6v1e1*,

which lies 7 Mb upstream of *pi6*, more than doubled in *pi6* mutants (2.1- and 2.3-fold increase in $pi6^{em1/em1}$ and $pi6^{em2/em2}$ spermatids, respectively), but expression of intervening genes was unaltered. Among the genes between *pi6* and *Apt6v1e1*, 19 have mRNAs with abundance >10 molecules per cell in spermatids; none were affected by loss of *pi6* transcription. The widespread preservation of normal mRNA abundance for genes on chromosome 6 strongly argues that loss of *pi6* transcription has little or no effect on the chromatin structure of neighboring genes.

Genes Essential for Sperm Functions Are Regulated by *pi6* piRNAs

Ceacam2 encodes CEACAM2-L, a testis-specific isoform of the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) family of proteins. CEACAM2-L appears in elongated spermatids and becomes undetectable in epididymal sperm, suggesting a role as a cell surface, testicular cell adhesion factor[6]. *Pou2f2* encodes a transcription factor that binds DNA cooperatively with other POU domain-containing proteins[7,8]; *Pou2f2* is normally expressed in pre-meiotic, type-A spermatogonia, but not in gonocytes, meiotic germ cells, or post-meiotic germ cells[8]. The function of *Tcp11x2*, which encodes an X-linked T-complex 11 protein, is suggested by its well-characterized paralog, TCP11, a receptor for fertilization-promoting peptides that facilitate sperm capacitation[9–11].

pi6-Regulated Genes Regulate Related Cellular Processes

Seventeen additional genes whose mRNA abundance increased in *pi6* mutants have reported functions only in somatic cells; the functions of two (*Gm595* and *2010003K11Rik*) are unknown (Supplementary Table 3). Loss-of-function phenotypes have been reported for 15 of the 24 *pi6* piRNA repressed genes. Knockout of nine genes (*Atp6v1e1*, *Rtn4*, *Ctnna2*, *Pskh1*, *Sacm1l*, *Dnajc3*, *Pouf2f2*, *Dcaf13*, *Fth1*) leads to embryonic or neonatal lethality or premature death in mice (Supplementary Table 3).

Two-thirds of the 24 genes encode subunits of multi-protein complexes with known functions. Overexpression of individual subunits can disrupt the function of a complex by a variety of mechanisms[12]. Therefore, although in vivo knockout studies have not been reported for many *pi6*-regulated genes, their functions can be inferred from that of the larger complex in which they reside. For example, loss of THOC1 or THOC5 in the TREX complex, which also contains THOC7 and ALYREF/THOC4, or of TMED2, which forms a complex with TMED9, causes embryonic lethality[13–15]. For 12 of the *pi6*-regulated genes, knockout mutants die before puberty, preventing assessment of a role in male fertility using existing alleles. Heterozygous mutants of one of these homozygous lethal genes, *Sacm1l*, have abnormal testis or epididymis morphology, suggesting that *Sacm1l* is important for spermatogenesis[16].

In addition to ATP6V1E1 and CATSPERE1, eight *pi6* piRNA-regulated genes encode integral membrane proteins or regulators of membrane biogenesis or function (*Cdh9*, *Ceacam2, Dnajc3*, *Kctd7*, *Rtn4*/*Nogo*, *Sacm1l*, *Tmed9*, and *Piro-1*/Gm10800)[17–26]. These proteins could play a role in acrosome function. Another 8 *pi6*-regulated genes function in pathways that control or respond to intracellular ion levels. *Catspere1*, *Cdh9*, *Ceacam2*, *Prkd2*, *Pskh1*, and *Rtn4* encode proteins that regulate or respond to intracellular Ca^{2+} concentration[17,23,25,27–32]. The protein products of *Atp6v1e1* and *Fth1* are required for intracellular H^{+} and Fe^{3+} homeostasis, respectively[33–35]. Thus, altered ion homeostasis may explain at least some of the defects of *pi6* mutant sperm.

Three *pi6*-regulated genes encode proteins that function in proteasomal protein degradation (KCTD7, DCAF13*,* DNAJC3). Four mediate cell-cell adhesion (CDH9, CTNNA2 [αN-Catenin], CEACAM2, PRKD2). Two, ALYREF/THOC4 and THOC7, act in mRNA processing or export as components of the TREX complex, which couples mRNA transcription, splicing, and nuclear export[36–38].

Validation of Direct mRNA Targets of *pi6* piRNAs

To validate targets predicted to be cleaved by *pi6* piRNAs, we used qRT-PCR to measure the abundance of full length mRNA in wild-type and $pi6^{em1/em1}$ spermatids using primers spanning the putative cleavage site (Extended Data Fig. 5 and 6a). Consistent with our RNA-seq data, all six *pi6* piRNA target mRNAs increased >3-fold in mutant spermatids ($p \leq 0.03$, $n = 3$; Extended Data Fig. 6a).

Examples of mRNA Targets Cleaved by Specific *pi6* piRNAs

For example, a target cleavage site in *Catspere1* mRNA exon 11 is complementary to nucleotides g2–g7 and to an additional 11 nucleotides between g8 and g21 of a *pi6* piRNA present at 744 molecules per cell in diplotene spermatocytes ($n = 3$). In $pi6^{em1/em1}$ pachytene, diplotene, and secondary spermatocytes, we could no longer detect the 5′ monophosphorylated 3′ product of *Catspere1* mRNA cleavage, and the steady-state abundance of *Catspere1* mRNA increased 2.1- (FDR = 5×10^{-18}) and 2.2- (FDR = 6×10^{-8}) times in $pi6^{em1/em1}$ and $pi6^{em2/em2}$ spermatids, respectively (Fig. 6b, Extended Data Fig. 6a, and Supplementary Table 2). We detected similar *pi6*-dependent cleavage sites in *Alyref* (one open-reading frame [ORF] cleavage site in exon 5), *Dnajc3* (one ORF cleavage site in exon 5), *Fth1* (one ORF cleavage site in exon 2; one ORF cleavage site in exon 4), *Kctd7* (two 3′ UTR cleavage sites in exon 4), and *Scpep1* (one ORF cleavage site in exon 4)

pi6 piRNAs Initiate piRNA Biogenesis from Two Pachytene piRNA Loci on Chromosome 10

Loss of *pi6* piRNAs had no significant effect (>2 fold-change and FDR <0.05) on the abundance of mRNAs encoding piRNA pathway proteins or piRNAs derived from major piRNA-producing loci (Extended Data Fig. 3b and 6b; Supplementary Table 5). At their peak expression in wild-type diplotene spermatocytes, *pi10-qC2-545.1*

(*chr10:94,673,712–94,688,442*) produces 0.15 ± 0.01% (26,192 molecules per diplotene spermatocyte, n = 3) of pachytene piRNAs, while *pi10-qA3-143.1* (*chr10:20,310,505–20,312,312*) produces 0.0259 ± 0.0003% (4,470 molecules per diplotene spermatocyte, n = 3). In *pi6* mutants, *pi10-qC2-545.1* piRNAs decreased 2.5-fold in pachytene spermatocytes (p = 0.005) and ≥3-fold in diplotene spermatocytes, secondary spermatocytes, and spermatids (p = 0.0001). *pi10-qA3-143.1* piRNAs declined 1.7-fold in $pi6^{em1/em1}$ diplotene spermatocytes (p = 0.0001) and 1.5-fold in secondary spermatocytes (p = 0.007), and spermatids (p = 0.0005). A corresponding increase in piRNA precursor transcripts accompanied the decrease in piRNA abundance: in both $pi6^{em1/em1}$ and $pi6^{em2/em2}$ mutants, *pi10-qC2-545.1* precursors increased 2.1–3-fold in pachytene spermatocytes and diplotene spermatocytes (FDR = 3×10^{-13} to 6×10^{-5}; Fig. 7c and Supplementary Table 2). Similarly, the abundance of *pi10-qA3-143.1* precursor transcripts increased 2–2.7-fold in diplotene spermatocytes, secondary spermatocytes, and spermatids (FDR = 4×10^{-26} to 2×10^{-10}) in the two *pi6* mutant alleles.

pi6 piRNAs Cleave *pi10-qC2-545.1* Precursor Transcripts

When cut by a *pi6* piRNA (11,000 ± 1000 molecules per cell; n = 3) in wild-type diplotene spermatocytes, RNA fragments derived from a cleavage site at *chr10:94,675,047–94,675,048* decreased >70-fold in $pi6^{em1/em1}$ diplotene spermatocytes (n = 2). For *pi10-qA3-143.1*, we identified a *pi6* piRNA-dependent cleavage site that produces 5′ monophosphorylated RNA in wild-type pachytene (3.3 ppm, n = 2) and diplotene spermatocytes (4.8 ppm, n = 2) but not in $pi6^{em1/em1}$ cells.

Reciprocal Initiation of piRNA Biogenesis Is Shared Among Most Pachytene piRNA Loci

Among the 22 loci that produce the most pachytene piRNAs in mice, 18 show such reciprocal piRNA-directed piRNA precursor cleavage. Four of these 18 loci (*7-qD2-11976.1*, *7-qD2-24830.1*, *pi17-qA3.3-26735.1,* and *pi17-qA3.3-27363.1*) generate piRNAs that reciprocally target the opposite arm of their shared promoter. Another four loci (*7-qD1-19431.1*, *9-qC-31469.1*, *12-qE-23911.1*, and *15-qD1-17920.1*) produce piRNAs that cleave their own precursors.

pi6 piRNAs Initiate piRNA Biogenesis from Protein-coding Genes

The *Kctd7* and *Fth1* loci had been previously annotated as sources of hybrid (*pi-Kctd7*, *chr5: 130,144,861–130,155,806*) or pre-pachytene (*pi-Fth1*, *chr19:9,982,703–9,985,092*) piRNAs. Like many piRNA-producing mRNAs, *Kctd7* produces piRNAs (9,700 ± 400 molecules per diplotene spermatocyte, $n = 3$) from its 3′ UTR. In contrast, *Fth1* piRNAs (160 ± 10 molecules per diplotene spermatocyte, $n = 3$) derive from the exons and 5′ UTR of the mRNA. In $pi6^{em1/em1}$ diplotene spermatocytes and secondary spermatocytes, the abundance of *Kctd7* piRNAs declined 5.3-fold ($p < 0.0001$, $n = 3$; Fig. 7b), while the *Kctd7* and *Fth1* transcripts more than doubled in *pi6* mutants (Fig. 7c and Supplementary Table 2). Further supporting the idea that *pi6* piRNAs initiate piRNA biogenesis from these two protein-coding loci, our data identify two *pi6* piRNA-directed cleavage sites in the *Kctd7* 3′ UTR and two sites in *Fth1* mRNA exons (exon 2 and exon 4; Supplementary Table 4).

Supplementary Discussion

Functional Redundancy of Pachytene piRNAs

The phenotypic and molecular specificity of *pi6* mutants likely reflects a low degree of redundancy with other piRNA-producing loci. Nonetheless, other piRNA-producing loci may partially rescue loss of *pi6* piRNAs, accounting for the incomplete penetrance of the *pi6* sterility phenotype. Conversely, the lack of a phenotype for other pachytene piRNA-producing loci may simply reflect greater redundancy with their piRNA-producing peers. In this view, loss of regulation of the targets of *pi17* piRNAs may be compensated by piRNAs from other loci. Testing this hypothesis is clearly a prerequisite to explain why loss of *pi6* and not *pi17* piRNAs has an obvious biological consequence. Indeed, genetic evidence supports the hypothesis that pachytene piRNAs from other piRNA-producing loci are functionally redundant with piRNAs from *pi17* (P.-H.W, K.C., and P.D.Z., unpublished data).

A Role for Pachytene piRNAs in Translational Activation

Recently, a subset of pachytene piRNAs has been proposed to activate translation[39]. In this model, pachytene piRNAs are envisioned to bind sites in the 3′ UTRs of target mRNAs through as few as 7 nt of seed complementarity (g2–g8). By comparison, our study of cleaved fragments derived from the RNA targets of *pi6* piRNAs in *pi6* mutant mice supports an siRNA-like cleavage mechanism that requires extensive base-pairing between piRNAs and target sites, most of which lie in the coding sequence of their target mRNAs. Our data suggest that the piRNA:mRNA base-pairing scheme proposed to activate translation activation is unlikely to trigger target cleavage. Although no *pi6* piRNA has been suggested to activate translation, two abundant *pi17* piRNAs were reported to activate translation of *Tbpl1* and *Cnot4*. We detect 9,000 ± 2,000 molecules (~7 nM) of the *Tbpl1*-targeting piRNA and 5,000 ± 500 molecules (~4 nM) of the *Cnot4*-targeting piRNA per individual diplotene spermatocyte (n = 3). Their corresponding targets, *Tbpl1* and *Cnot4*, are present at 319 and 80 molecules per diplotene spermatocyte. Although the intracellular concentration of the piRNAs is sufficient to

support a high occupancy of their predicted targets in the absence of other RNAs, the overall abundance of 7mer seed (g2–g8) matches in the transcriptome of diplotene spermatocytes vastly exceeds the number of molecules of either of these two piRNAs: for the *Tbpl1*-targeting piRNA, there are ~45 times more sites than piRNA (~392,800 sites per diplotene spermatocyte, roughly half in 3′ UTRs), and for the *Cnot4*-targeting piRNA ~114 times more sites than piRNA (607,600 molecules per diplotene spermatocyte, roughly 13% in 3′ UTRs). We cannot envision a mechanism by which binding of a piRNA can promote the translation of a specific mRNA in the presence of such a vast excess of essentially identical binding sites. Moreover, the absence of a phenotype in *pi17* $^{-/-}$ mice suggests that translational activation of *Tbpl1* and *Cnot4* by these piRNAs is dispensable for the successful completion of both spermatogenesis and spermiogenesis.

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