The evolutionarily conserved piRNA-producing locus pi6 is required for male mouse fertility

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Pachytene PIWI-interacting RNAs (piRNAs), which comprise >80% of small RNAs in the adult mouse testis, have been proposed to bind and regulate target RNAs like microRNAs, cleave targets like short interfering RNAs or lack biological function altogether. Although piRNA pathway protein mutants are male sterile, no biological function has been identified for any mammalian piRNA-producing locus. Here, we report that males lacking piRNAs from a conserved mouse pachytene piRNA locus on chromosome 6 (pi6) produce sperm with defects in capacitation and egg fertilization. Moreover, heterozygous embryos sired by pi6−/+ fathers show reduced viability in utero. Molecular analyses suggest that pi6 piRNAs repress gene expression by cleaving messenger RNAs encoding proteins required for sperm function. pi6 also participates in a network of piRNA–piRNA precursor interactions that initiate piRNA production from a second piRNA locus on chromosome 10, as well as pi6 itself. Our data establish a direct role for pachytene piRNAs in spermiogenesis and embryo viability.

Only animals produce PIWI-interacting RNAs (piRNAs), 21–35-nucleotide RNAs that form the most abundant small RNA class in the germline. piRNAs protect the germline genome from transposons and repetitive sequences, and, in some arthropods, fight viruses and transposons in somatic tissues. The mammalian male germline makes three classes of piRNAs: (1) 26–28-nucleotide transposon-silencing piRNAs predominately in the fetal testis; (2) shortly after birth, 26–27-nucleotide piRNAs derived from mRNA 3′ untranslated regions (UTRs) emerge; and (3) at the pachytene stage of meiosis, ~30-nucleotide, non-repetitive pachytene piRNAs appear. Pachytene piRNAs accumulate to comprise >80% of all small RNAs in the adult mouse testis, and they continue to be made throughout the reproductive lifespan of the male mouse. These piRNAs contain fewer transposon sequences than the genome as a whole, and most pachytene piRNAs map only to the loci from which they are produced. The diversity of pachytene piRNAs is unparalleled in development, with >1 million distinct species routinely detected in spermatocytes or spermatids. Intriguingly, the sequences of pachytene piRNAs are not themselves conserved, but piRNA-producing loci have been maintained at the syntenic regions of chromosome 6 for millions of years across eutherian mammals, suggesting that the vast sequence diversity of pachytene piRNAs is itself biologically meaningful.

One hundred mouse pachytene piRNA-producing loci have been annotated. All are coordinately regulated by the transcription factor A-MYB (MYBL1), which also promotes expression of proteins that convert piRNA precursor transcripts into mature piRNAs, as well as proteins required for cell cycle progression and meiosis. Of the 100 pachytene-producing loci, 15 pairs of pachytene piRNA-producing genes are divergently transcribed from bidirectional, A-MYB-binding promoters. The contribution of pachytene piRNAs from each piRNA-producing locus is unequal, with just five loci located on five different chromosomes—pi2, pi6, pi7, pi9 and pi17—contributing >50% of all pachytene piRNAs at 17 d postpartum (dpp).

Loss of proteins required to make pachytene piRNAs, including the pachytene piRNA-binding protein MIWI (PIWIL1), invariably arrests spermatogenesis without producing sperm, thus rendering males sterile. Yet, loss of the chromosome 17 pachytene piRNA-producing locus, 17-qA3.3–27363(−),26735(+) (henceforth, pi17), has no detectable phenotype or impact on male fertility, even though pi17 produces ~16% of all pachytene piRNAs in pachytene spermatocytes. Similarly, mice with disrupted expression of a chromosome 2 piRNA locus are viable and fertile (P.-H.W., K.C. and P.D.Z, unpublished observations, and ref. 21). Consequently, the function of pachytene piRNAs in mice is actively debated. One model proposes that pachytene piRNAs regulate meiotic progression of spermatocytes by cleaving mRNAs during meiosis. Another model posits that pachytene piRNAs direct degradation of specific mRNAs via a miRNA-like mechanism involving mRNA deadenylation. A third model proposes that MIWI functions without piRNAs, and that piRNAs are byproducts without a critical function. Compelling evidence supports each model.

In fact, direct demonstration of piRNA function in any animal has proven elusive. Only two piRNA-producing loci have been directly shown to have a biological function; both were identified genetically before the discovery of piRNAs and are found only in members of the melanogaster subgroup of flies. In male Drosophila melanogaster, piRNAs from Suppressors of Stellate, a multi-copy gene on the Y chromosome, silence the X-linked selfish gene Stellate; deletion of Suppressors of Stellate leads to Stellate protein crystals in spermatocytes. In female flies, deletion of the piRNA-producing flamenco gene, which is expressed in somatic follicle cells that support oogenesis, leads to gypsy family transposon activation and female infertility.

Here, we report that a promoter deletion in the chromosome 6 pachytene piRNA locus 6-qF3–28913(−),8009(+) (chr6:127,776,075–127,841,890, mm10; henceforth, pi6) disrupts
male fertility. The pi6 locus generates 5.8% of pachytene piRNAs in the adult testis and is conserved among eutherian mammals. Mice lacking pi6-derived piRNAs produce normal numbers of sperm and continue to repress transposons. However, pi6 mutant sperm show defective capacitation and fail to penetrate the zona pellucida, a glycoprotein layer surrounding the egg. Consistent with these phenotypes, spermatids from pi6 mutant males show increased steady-state abundance of mRNAs encoding proteins involved in sperm acrosome function and penetration of the oocyte zona pellucida. In addition to decreasing specific mRNA abundance, pi6 piRNAs concurrently facilitate biogenesis of piRNAs from other loci. Our findings provide direct evidence for a biological function for pachytene piRNAs in male mouse fertility, and pi6 promoter deletions provide a model for future studies of piRNA biogenesis and function.

Results

pi6 promoter deletion eliminates pi6 pachytene piRNAs. To eliminate production of pi6 pachytene piRNAs while minimizing the impact on adjacent genes, we used Cas9 and a pair of single-guide RNAs (sgRNAs) to delete a 227-base-pair (bp) sequence that encompasses the A-MYB-binding site and promoter (ref. 10; Fig. 1, Extended Data Fig. 1a,b and Supplementary Table 1). To test that the phenotype of pi6em1/em1 male mice reflects loss of the pi6 promoter—and not an off-target mutation elsewhere in the genome—we used a second pair of sgRNAs to generate a 117-bp pi6 promoter deletion, pi6em2 (Fig. 1, Extended Data Fig. 1a,c and Supplementary Table 1). For comparison, we created an analogous 583-bp promoter deletion in pi17. We established stable pi6em2 mutant lines (pi6em2−1, −2 and −3 in Extended Data Fig. 1a,b) from three founders whose pi6 promoter deletion sizes range from 219 to 230bp and differ at their deletion boundaries, reflecting imprecise DNA repair after Cas9 cleavage. All three deletions eliminated pi6 primary transcripts and mature pachytene piRNAs from both arms of the locus (Fig. 1). Because these lines were created using the same pair of sgRNA guides, and we refer to all as the pi6em2 allele. Similarly, we refer to the stable mutant lines generated from mutant founders carrying pi6em2 (pi6em2−1 and −2 in Extended Data Fig. 1a,c) or pi17−/− (pi17−/−1 and −2 in Extended Data Fig. 1a,d) deletions as pi6em2 and pi17−/− alleles, respectively.

pi6 is required for male mouse fertility. When paired with C57BL/6 females, 2–8-month-old pi6em1/em1 males produced fewer pups compared with their littermates, even at peak reproductive age (Fig. 2a and Extended Data Fig. 2a). In 6 months, C57BL/6 males produced 7 ± 1 (n = 5) litters, while pi6em1/em1 males produced 2 ± 2 (n = 6) litters. The significantly smaller number of progeny produced by pi6em1/em1 males (P = 0.007 for all litters; P = 0.006 for viable litters only) over their reproductive lifetime reflects two abnormal aspects of their fertility (Fig. 2a,b and Supplementary Note). First, 29% of pi6em1/em1 males never produced pups. Second, the mutants that did sire pups did so less frequently. In contrast, males and females carrying an ~583-bp promoter deletion in pi17 were fully fertile, as observed previously for an independent, partial loss-of-function pi17 promoter deletion, despite loss of primary transcripts and mature piRNAs from both arms of the pi17 locus (Fig. 1).

Like pi6em1/em1 male mice, pi6em2/em2 males produced neither primary pi6 transcripts nor mature pi6 piRNAs and showed reduced fertility (Fig. 1 and Extended Data Fig. 2a). We conclude that pi6 piRNAs are required for male fertility in C57BL/6 mice.

pi6 mutant males produce fewer embryos. pi6 mutant male matings produced fewer fully developed embryos. We examined the embryos produced by natural mating of C57BL/6 females housed
with C57BL/6, pi6+em1, or pi6em2 males at 8.5, 14.5 or 16.5 d after occurrence of a mating plug. At 8.5 d after mating, C57BL/6 females housed with pi6em2 males carried fewer embryos (2 ± 2, n = 3) compared with females paired with pi6+em1 (6 ± 5, n = 2) or C57BL/6 control (7 ± 4, n = 1) males (Fig. 2c). At 14.5 and 16.5 d after mating, female mice paired with pi6em1 males had even fewer embryos. Female mice paired with pi6em2 males, similarly, had fewer embryos at 14.5 d after mating (2 ± 3, n = 3). Naturally born pups sired by pi6+em1 and pi6em2 males were rare but healthy, with no obvious abnormalities (Extended Data Fig. 2b and Supplementary Note).

**pi6 mutant sperm fail to fertilize wild-type eggs.** pi6+em1 and pi6em2 adult testes had normal gross histology (Fig. 2d, Extended Data Fig. 2b and Supplementary Note). The quantity of caudal epididymal sperm produced by pi6em1 mice (19 ± 10 million sperm per ml; n = 6) was also comparable to that of their pi6+em1 (23 ± 7 million sperm per ml; n = 4) or C57BL/6 (20 ± 10 million sperm per ml; n = 4) males (Fig. 2d).
per ml; n = 13) litermates (Fig. 2e, Extended Data Fig. 2c–e and Supplementary Note).

Because pi6−/− males are infertile at sire offspring, we used in vitro fertilization (IVF) to distinguish between defects in mating behavior and sperm function, incubating sperm from C57BL/6, pi6em1 or pi6em2 males with wild-type oocytes and scoring for the presence of both male and female pronuclei and the subsequent development of the resulting bi- androgenic zygotes into two-cell embryos 24 h later (Fig. 3a). The majority of oocytes incubated with sperm from C57BL/6 (86 ± 17%; 774 total oocytes; n = 6) or pi6em1 (60 ± 35%; 412 total oocytes; n = 5) males developed into two-cell embryos. By contrast, only 7 ± 5% (12-fold decrease compared to C57BL/6; Cohen’s d = 6.3; 1,026 total oocytes; n = 7) of oocytes incubated with pi6em2 sperm reached the two-cell stage. Similarly, no oocytes incubated with pi6em2 sperm developed into two-cell embryos by 24 h. The majority of these oocytes remained undivided, and few contained a male pronucleus, suggesting that pi6em1 and pi6em2 sperm are defective in fertilization.

pi6em1 and pi6em2 sperm nuclei support fertilization. The best-studied piRNA function is transposon silencing, and mice pi2 have been proposed to be involved in LINE1 element silencing, although pi2 mutant males are fertile31. Moreover, LINE1 transcript abundance increases in mice bearing inactivating mutations in the catalytic site of MIWI44. Transposon activation can produce DNA damage, and genomic integrity is critical for fertilization45–47. However, pachytene piRNAs are depleted of repetitive sequences, in contrast to other types of piRNA-producing genomic loci (Extended Data Fig. 3a–c, d).

We asked whether the defect in fertilization by pi6 mutant sperm might reflect DNA damage or epigenetic dysregulation of the sperm genome. pi6em1 or pi6em2 sperm heads were individually injected into the cytoplasm of wild-type oocytes (intracytoplasmic sperm injection, ICSCI; Fig. 3b), bypassing the requirement for sperm motility, acrosome reaction, egg binding or sperm–egg membrane fusion48. pi6em1 sperm heads delivered by ICSCI fertilized the oocyte at a rate similar to that of pi6em1 sperm: 66% of oocytes (161 total viable oocytes from two separate trials) injected with homozygous mutant pi6em1 sperm heads reached the two-cell stage, compared to 79% for pi6em1 sperm (61 total viable oocytes from two separate trials). Thus, most pi6em1 nuclei are capable of fertilization. We found no DNA damage or increased transposon expression in pi6em1 and pi6em2 spermatogenic cells, further evidence that indicates pi6 is not important for transposon silencing (Extended Data Fig. 3b, c and Supplementary Note).

pi6 mutant sperm struggle to penetrate the zona pellucida. Mammalian spermatozoa stored in the epididymis are immotile and dormant. Sperm capacitate, that is, resume maturation, only upon entering the female reproductive tract49. Upon capacitation, sperm become capable of undergoing the acrosome reaction, which is required to bind and penetrate the outer oocyte glycoprotein layer, the zona pellucida44–46. To test whether the defect in fertilization by pi6 mutant sperm reflects impaired binding to or penetration of the zona pellucida, we compared IVF using unmanipulated oocytes to oocytes with the zona pellucida removed (Fig. 3a). Strikingly, removing the zona pellucida fully rescued the fertilization rate of pi6 em1 sperm: 92 ± 7% (316 total oocytes; n = 3) of zona pellucida-free oocytes incubated with pi6em1 sperm reached the two-cell stage after 24 h, compared to 7 ± 5% for intact zona pellucida (1,026 total oocytes; n = 3). Similarly, 98% of zona pellucida-free oocytes incubated with pi6em2 sperm developed into two-cell embryos after 24 h (98 total oocytes; n = 1), in contrast to 0% of those with intact zona pellucida (140 total oocytes; n = 1).

Impaired capacitation in pi6 mutant sperm. One hallmark of sperm capacitation is a switch to ‘hyperactivated motility’, a swimming pattern characterized by a high amplitude and nonsymmetric beating of the flagellum that facilitates penetration of the zona pellucida5–30. To assess pi6 mutant sperm capacitation, we measured the motility of freshly extracted caudal epididymal sperm from pi6em1, pi6em2 or C57BL/6 mice using computer-assisted sperm analysis (CASA41; Fig. 4a). After 90 min incubation under capacitation-promoting conditions, pi6em1 and pi6em2 sperm populations had reduced path and progressive velocity, which are measures of sperm motility, compared to control sperm (Fig. 4b, c, Supplementary Videos 1–10 and Supplementary Note).

To more rigorously evaluate progressive motility and hyperactivation, we used CASAnova, an unsupervised machine learning tool12, to analyze caudal epididymal sperm from pi6em1, pi6em2 and C57BL/6 control mice. After 90 min in capacitating conditions, CASAnova identified just 0.3 ± 0.5% of pi6em1 (n = 11) and 0.2 ± 0.3% of pi6em2 (n = 2) sperm as progressive, compared to 9 ± 7% for C57BL/6 (n = 9; Fig. 4d). Similarly, only 2 ± 1% of pi6em1 or pi6em2 sperm displayed hyperactivated motility, compared to 8 ± 2% for the control (Fig. 4d), a percentage that is typical for the C57BL/6 mouse strain32.

Acrosome reaction in sperm can be visualized and measured ex vivo (Fig. 4a, c). While the spontaneous acrosome reaction rates for C57BL/6 (18 ± 3%; n = 5) and pi6 mutant sperm were similar (15 ± 6%; n = 5), acrosome reaction triggered by ionophore-induced Ca2+ influx (that is, ionophore-induced minus spontaneous) differed between the two genotypes: 46 ± 10% (±12%) of pi6 mutant sperm (n = 5) underwent partial or complete reaction, compared to 68 ± 6% (50 ± 7; n = 5) for C57BL/6 (P = 0.01; Cohen’s d = 2.78; Fig. 4e). Our data suggest that pi6 mutant sperm less effectively undergo an acrosome reaction triggered by ionophore-induced Ca2+ influx, a defect expected to impair binding and penetrating of the zona pellucida. Together, our data indicate that insufficient capacitation is responsible for the poor fertilization capability of pi6 mutant sperm.

Potential role of paternal pi6 piRNAs in embryo development. Even when pi6 mutant sperm successfully fertilize an oocyte, the resulting heterozygous embryos are less likely to complete gestation. We monitored preimplantation development in vitro for up to 96 h, a period during which the one-cell embryo develops into a blastocyst. Of the oocytes incubated with pi6em1 sperm, 40% remained undivided without evidence of a male pronucleus, presumably because they were not fertilized. Among the remaining 60% oocytes that progressed to at least the two-cell stage, indicating successful fertilization by pi6em1 sperm, 82% showed delayed development, requiring 48 h to reach the two-cell stage. None of these developed further. Just 3% of oocytes fertilized by pi6em1 sperm progressed to the blastocyst stage by 96 h, compared to 98% for C57BL/6 sperm (Fig. 5a).

Two-cell embryos generated by IVF using sperm from pi6em1, pi6em2 or C57BL/6 control mice were transferred to wild-type surrogate mothers (Fig. 5b). Most embryos from pi6em1 (50 ± 10%; 23 ± 4 embryos per female; n = 3) or C57BL/6 control sperm (70 ± 10%; 21 ± 3 embryos per female; n = 3) developed to term (Fig. 5c, Extended Data Fig. 4a and Supplementary Note), a rate typical for this genetic background31. By contrast, only 20 ± 20% of two-cell embryos from pi6em1 sperm developed to term (n = 6). Likewise, fewer ICSI-derived pi6em1 embryos developed to term in the surrogate females (Fig. 5d, Extended Data Fig. 4b and Supplementary Note). We conclude that paternal pi6 piRNAs play a direct or indirect role in early embryogenesis.

Changes in spermatocyte and spermatid mRNA abundance accompany loss of pi6 piRNAs. Pachyteme piRNAs repress their RNA targets at least in part by an siRNA-like cleavage mechanism. Mice bearing mutations that selectively inactivate the endonuclease activity of MIWI are phenotypically indistinguishable from those
lacking MIWI altogether\textsuperscript{15,16}. Moreover, ectopic expression in mice of the largest human piRNA-producing locus triggers cleavage and degradation of mouse \textit{Dpy19l2} mRNA, causing male sterility\textsuperscript{22}. To begin to identify direct targets of \textit{pi6} piRNAs, we used RNA-seq to measure steady-state RNA abundance in pachytene spermatocytes, diplotene spermatocytes, secondary spermatocytes and spermatids purified from \textit{pi6}\textsuperscript{em1/em1}, \textit{pi6}\textsuperscript{em2/em2} and C57BL/6 adult testis (Fig. 6a).

The steady-state abundance of the RNA targets of \textit{pi6} piRNA-guided cleavage are predicted to increase in \textit{pi6} mutants. We searched for transcripts whose steady-state abundance increased in both \textit{pi6}\textsuperscript{em1/em1} (\(n = 4\)) and \textit{pi6}\textsuperscript{em2/em2} (\(n = 3\)) cells compared to C57BL/6 controls (\(n = 4\); Fig. 6b and Supplementary Table 2). \textit{pi6}\textsuperscript{em1} and \textit{pi6}\textsuperscript{em2} deletions increased the abundance of 8 diplotene spermatocyte mRNAs, 15 secondary spermatocyte mRNAs and 21 spermatid

**Fig. 3 | Fertilization defects of \textit{pi6}\textsuperscript{em1/em1} and \textit{pi6}\textsuperscript{em2/em2} sperm revealed by IVF and ICSI.** \textbf{a}, Sperm function analyzed by IVF using oocytes with or without zona pellucida (ZP). Vertical black lines denote median; boxes indicate 75th and 25th percentiles; whiskers report the maximal and minimal values. Each dot represents the IVF result using sperm from an individual male. \textbf{b}, Sperm function analyzed by ICSI. See also Extended Data Fig. 3.
mRNAs, but did not affect genes neighboring pi6 (Supplementary Note). Although pi6 piRNAs first begin to accumulate in pachytene spermatocytes (Extended Data Fig. 3b), the abundance of no pachytene spermatocyte mRNA changed significantly (false discovery rate (FDR) < 0.05) in both pi6em1 and pi6em2 mice, suggesting that pi6 piRNAs do not accumulate to functional levels until the diplotene phase of meiosis. In total, loss of pi6 piRNAs more than doubled the mRNA level of 24 genes in at least one spermatogenic cell type, 13 (54%) of which remained significantly altered in subsequent stages.

**Genes essential for sperm functions are regulated by pi6 piRNAs.** Among the 24 genes with increased mRNA abundance in pi6 mutant cells, Atp6v1e1 and Catsper1 encode proteins required for sperm capacitation.
Fig. 5 | Embryos derived from \( p<sub>6</sub><sup>em1</sup>/em1 \) sperm fail to develop. **a**, Development of IVF-derived embryos. Red, number of embryos that developed to the stage appropriate for the elapsed time after fertilization. **b**, Strategy for surgical transfer of fertilized two-cell embryos to surrogate mothers. **c**, Percentages of IVF-derived two-cell embryos using sperm from C57BL/6 \((n=3)\), \( p<sub>6</sub><sup>em1</sup>/em1 \) \((n=3)\) or \( p<sub>6</sub><sup>em1</sup>/em1 \) \((n=6)\) mice that developed to term. Each uterine cartoon represents one surrogate mother; colored circles depict embryos. The number of embryos transferred to each side of the oviduct is indicated. N/A, not applicable. **d**, Percentages of ICSI-derived two-cell embryos using sperm from \( p<sub>6</sub><sup>em1</sup>/em1 \) \((n=2)\) or \( p<sub>6</sub><sup>em1</sup>/em1 \) \((n=2)\) mice that developed to term. See also Extended Data Fig. 4.

### Embryos derived from \( p<sub>6</sub><sup>em1</sup>/em1 \) sperm fail to develop

| Sperm donor genotype | Trial | Number and placement of two-cell embryos in surrogate mother | Percentage developed to live fetus |
|----------------------|-------|---------------------------------------------------------------|----------------------------------|
| C57BL/6              | 3     | 12 12 12 12                                                 | 60 N/A                           |
|                      | 4     | 9 9 9 9                                                     | 72 N/A                           |
|                      | 6     | 10 10                                                      | 85 N/A                           |
|                      |       | Mean ± s.d. 70 ± 10                                            | N/A                              |
| \( p<sub>6</sub><sup>em1</sup>/em1 \) | 1     | 12 12 12 12 12                                               | 54 N/A                           |
|                      | 2     | 12 12 12 12 12                                               | 39 N/A                           |
|                      | 3     | 12 12 12 12 12                                               | 58 N/A                           |
|                      |       | Mean ± s.d. 50 ± 10                                            | N/A                              |
| \( p<sub>6</sub><sup>em1</sup>/em1 \) | 1     | 0 5 7 9                                                     | 0 N/A                            |
|                      | 2     | 0 7 9 12                                                   | 0 N/A                            |
|                      | 3     | 0 12 12                                                    | 50 33                            |
|                      | 4     | 8 9 9 9 9 9                                               | 0 39                             |
|                      | 5     | 10 10 10                                                   | 40 80                            |
|                      | 6     | 10 10 10                                                   | 15 N/A                           |
|                      |       | Mean ± s.d. 20 ± 20                                             | 50 ± 20                           |
**Fig. 6** | mRNAs encoding proteins required for sperm capacitation and zona pellucida binding are direct targets of pi6 piRNAs. **a,** Strategy for purifying specific male germ cell types. **b,** Scatter plots of steady-state transcript abundance in sorted testicular germ cells. Each dot represents the mean abundance of an mRNA measured using four (wild-type and pi6 em1/em1) cells or three (pi6 em2/em2) individual males. Differentially expressed transcripts (>2 fold change and FDR <0.05) were identified using DESeq2 (see also Methods) and are indicated. **c,** Ultrastructure of caudal epididymal sperm flagella and acrosomes from mice of indicated genotypes by transmission electron microscopy. See also Extended Data Figs. 5 and 6 and Supplementary Tables 2, 3 and 4.
pi6 piRNAs direct cleavage of their mRNA targets. All known catalytically active Argonaute proteins cleave their targets at the phosphodiester bond linking nucleotides t10 and t11, the bases paired to guide nucleotides g10 and g11. Target cleavage generates a 5′ product bearing a 3′ hydroxyl terminus, and a 3′ product beginning with a 5′ monophosphate. Thus, cleaved targets can be identified by high-throughput sequencing methods designed to capture long RNAs bearing a 5′ monophosphate (degradome-seq), coupled with computational identification of piRNAs capable of directing production of the putative 3′ cleavage products.

We performed small RNA-seq to define the piRNA repertoire and degradome-seq of C57BL/6 and pi6 mice germ cells to identify candidate pi6 piRNA-directed target cleavage products. Because the specific rules for piRNA-guided target cleavage are poorly defined, we identified target candidates by first requiring g2–g7 seed complementarity between a pi6 piRNA and a cleaved RNA fragment. Then, we searched for seed-matched transcripts with a cleavage product whose 5′ end overlapped 10 nucleotides with the piRNA (Extended Data Fig. 5a). Finally, we compared both the steady-state (RNA-seq) and cleaved-fragment (degradome-seq) abundance of target candidate RNAs in wild-type and pi6 mutant germ cells. These criteria identified pi6 piRNA-dependent cleavage sites in six mRNAs whose abundance increased in pi6 mutants: Alyref, CatSper1, DnaJc3, Fth1, Kctd7 and Scpep1 (Extended Data Fig. 5b, Supplementary Table 4 and Supplementary Note).

pi6 piRNAs reciprocally facilitate biogenesis of piRNAs from other loci. Because piRNA-directed cleavage of piRNA precursor transcripts generates 5′ monophosphorylated pre-pre-piRNAs, piRNAs play a central role in the initiation of piRNA production. Consistent with this, pi6 piRNAs initiate piRNA production by cleaving pachytene piRNA precursor transcripts. In pi6 mice diplo- tene spermatocytes, we detected a large reduction in the abundance of 3′ cleavage fragments from pachytene piRNA precursor transcripts targeted by pi6 piRNAs, but not for piRNA precursor transcripts targeted by pi17 piRNAs (Fig. 7a, left panel). Strings of head-to-tail piRNAs (‘phased’ or ‘trailing’ piRNAs) beginning at the 5′ end of a piRNA-directed 3′ cleavage fragment are the hallmark of piRNA-initiated piRNA production. We detected such phased piRNAs downstream of pi6 piRNA-directed cleavage sites within pachytene piRNA precursors in wild-type diplo- tene spermatocytes (Fig. 7a, top right). In pi6 mutants, the abundance of these trailing piRNAs decreased, whereas the abundance of trailing piRNAs initiated by pi17 piRNAs was unchanged. Conversely, in pi17−/− mutants, the abundance of trailing piRNAs initiated by pi17 piRNAs, but not pi6 piRNAs, diminished (Fig. 7a, bottom right), indicating that initiation of piRNA production by piRNAs is not unique to pi6 piRNAs.

Intriguingly, loss of pi6 piRNAs specifically decreased the abundance of piRNAs and accumulated steady-state precursors from two pachytene piRNA-producing loci on chromosome 10, but not from any other loci, including the major piRNA loci pi2, pi7, pi9 or pi17 (Fig. 7b,c, Extended Data Figs. 3b and 6b, Supplementary Table 5 and Supplementary Note). Further supporting the idea that pi6 piRNA-directed cleavage initiates pachytene piRNA production from pi10-qC2-545.1, degradome sequencing detected two different pi6 piRNA-dependent cleavage sites in pi10-qC2-545.1 transcripts (Extended Data Fig. 5b and Supplementary Table 4). Each cleavage site can be explained by an extensively complementary pi6 piRNA predicted to direct MILI or MIWI to cleave the piRNA and a cleaved RNA fragment. Then, we searched for seed transcripts with a cleavage product predicted to direct MILI or MIWI to cut the pi10-qC2-545.1 precursor transcript immediately before the 5′ end of the 5′ monophosphorylated RNA identified by degradome sequencing. Remarkably, pi10-qC2-545.1 piRNAs, whose production required pi6 piRNAs, reciprocally promote pi6 piRNA biogenesis: three pi10-qC2-545.1 piRNAs map to pi6 precursor cleavage sites that initiate pi6 piRNA production in wild-type pachytene and diplo- tene spermatocytes (Extended Data Fig. 5c and Supplementary Table 4). pi10-qC2-545.1 is found in both mice and rats, but the gene produces a long non-coding RNA (IncRNA) in rats and a piRNA precursor in mice. The finding that pi10-qC2-545.1 generates piRNAs only in Mus musculus suggests that it emerged recently as a pachytene piRNA-producing locus. Perhaps the fortuitous production of pi6 piRNAs with sufficient complementarity to direct cleavage of pi10-qC2-545.1 transcripts has converted the locus to a source of piRNAs that enhance piRNA production from the more ancient pi6 locus.

Reciprocal initiation of piRNA biogenesis between pi6 and pi10-qC2-545.1 is far from exceptional. In fact, precursors from all major pachytene piRNA-producing loci are cleaved by pachytene piRNAs produced by another locus (Fig. 7d, Supplementary Table 6 and Supplementary Note). In addition to pachytene piRNA precursor targets, pi6 piRNAs also initiate piRNA biogenesis from two piRNA-regulated protein-coding genes, Kctd7 and Fth1 (Fig. 7b,c, Supplementary Tables 2 and 4 and Supplementary Note). Together, our data demonstrate that pi6 piRNAs not only repress mRNA expression, but also initiate piRNA biogenesis in trans from other piRNA-producing loci (Fig. 7e).

Discussion
Deletion of the promoter of the mouse pi6 pachytene piRNA locus causes specific, quantifiable defects in male fertility. These include impaired sperm capacitation and a failure of sperm to bind and penetrate the zona pellucida. The male fertility defects accompanying the loss of pi6 piRNAs are specific to this locus, as deletion of the promoter of pi17, which eliminates pi17 piRNAs, had no detectable effect on male or female fertility or viability, as reported previously. The phenotypic defects of pi6 mutants reflect the molecular changes—increased steady-state abundance of mRNAs that encode proteins functioning in sperm capacitation, acrosome function and other pathways with links to sperm biology.

Our finding that deletion of pi6, but not of pi17, the most prolific piRNA-producing locus, leads to male fertility defects, suggests that individual pachytene piRNA loci can regulate distinct sets of genes (Supplementary Note). Pachytene piRNAs have been proposed to act collectively in meiotic spermatocytes or postmeiotic spermatids to target mRNAs for destruction. Our data argue against pachytene
Fig. 7 | pi6 piRNAs and piRNAs from other loci form a network to repress mRNA expression and facilitate piRNA biogenesis. **a**. Cumulative abundance of pi6 and pi17 piRNA-directed, 3′ cleavage products in pi6em1 mouse (left panel). Analysis of 5′ to 5′ distances for mature piRNAs derived from pachytene piRNA precursors cleaved by pi6 or pi17 piRNAs in diploptene spermatocytes (right panel). piRNA-directed cleavage sites were identified requiring uninterrupted base pairing from position g2 to g16 between a pi6 (10 sites; top right panel) or pi17 (21 sites; bottom right panel) piRNA and the transcript, such that target cleavage between t10 and t11 would generate the 5′ monophosphorylated, 3′ cleavage fragment detected by degradome-seq. Pi value was computed using the Kolmogorov–Smirnov test. **b**. Abundance of mature piRNAs measured by small RNA-seq. Each dot represents the abundance of transcripts in one mouse. Pac., pachytene; Dip., diplotene; Sec., secondary. **c**. Expression of piRNA precursors measured by RNA-seq. Each dot represents the abundance of uniquely mapping reads in one mouse. Pac., pachytene; Dip., diplotene; Sec., secondary. **d**. A model for pi6 piRNA biogenesis and function. See also Extended Data Figs. 5 and 6 and Supplementary Tables 4, 5 and 6.
piRNAs acting en masse\textsuperscript{45,46}, since not only does pi6 produce far fewer piRNAs than pi17, but also just a tiny fraction of pi6 piRNAs can explain the effect of loss of pi6 piRNAs on the transcriptome. pi6 produces 80,354 distinct piRNA sequences, representing 10,943 unique g1–g21 piRNA sequences reproductibly (n = 3) present at >1 molecule per cell. Yet, loss of pi6 piRNAs dysregulates just 24 mRNAs, consistent with its remarkably specific mutant phenotype. This finding calls to mind the mechanism of sex determination in the silkworm Bombyx mori, where a single piRNA species derived from a piRNA precursor locus, Feminizer (Fem), on the W chromosome, targets the transcript of the Masculinizer gene on the Z chromosome. Among thousands of W chromosome-derived B. mori piRNAs, just a single piRNA species regulates the mRNA that plays a dominant role in female sex determination\textsuperscript{49}.

Moreover, our data argue strongly against miRNA-like regulation by piRNAs. miRNA binding through little more than the seed match accelerates mRNA degradation (ref. \textsuperscript{65} and Supplementary Note). siRNA-directed target cleavage requires more extensive sequence complementarity but can tolerate a variety of mismatches—even a seven-nucleotide insertion—around the cleavage site and at the 3’ end of the siRNA\textsuperscript{66}. If pachytene piRNAs bound their target RNAs by a miRNA-like, seed-based mechanism, the predicted target repertoire of piRNAs produced by individual loci would be enormous: pi6 piRNAs encompass 9,880 distinct 7mer–m8 seed sequences (g2–g8)\textsuperscript{49}, while pi17 generates 134,358 distinct piRNA sequences, encompassing 11,324 distinct g2–g8 seeds. Yet, loss of pi17 piRNAs has no detectable phenotype, while loss of pi6 piRNAs causes specific defects in sperm motility, the acrosome reaction and egg fertilization. Although 104 pi6 piRNAs are more abundant than miR-20a, the tenth most abundant miRNA in diploten spermatoocytes, loss of pi6 piRNAs reproducibly increases the abundance of just 24 mRNAs. Of these, just six mRNAs appear to be direct cleavage targets of pi6 piRNAs, consistent with pachytene piRNAs acting like long siRNAs: they find and cleave targets with extensive—but often incomplete—complementarity.

The current model for piRNA biogenesis posits that piRNA-directed cleavage of precursor transcripts facilitates the biogenesis of other piRNAs\textsuperscript{46,48}. Consistent with this, pi6 piRNAs are required for biogenesis of piRNAs from four other piRNA-producing loci: pi10-gC2-545.1 and pi10-gC2-143.1, both sources of pachytene piRNAs; and two protein-coding genes, Kdct7, a hybrid piRNA gene, and Fh1l, a pre-pachytene piRNA gene. Despite producing just 3% as many piRNAs as pi6, pi10-gC2-545.1 makes piRNAs that can cleave pi6 transcripts, initiating biogenesis of pi6 piRNAs. Such positive feedback loops appear to operate among many piRNA-producing loci, suggesting that this is an important mechanism for pachytene piRNA biogenesis in mice and perhaps other Eutheria. Given the strikingly small number of mRNAs regulated by pi6 piRNAs, we speculate that the extreme sequence diversity of pachytene piRNAs may be a natural byproduct of the positive feedback loops among pachytene piRNA loci. That is, many piRNAs may serve to reinforce piRNA production rather than reflect evolutionarily pressure to regulate large numbers of mRNAs.

Beyond the requirement for pi6 piRNAs to produce full functional sperm, pi6 piRNAs appear to play an additional role in embryo development. Our data suggest that the arrested development and reduced viability of embryos derived from pi6 mutant sperm reflects a paternal defect and not the embryonic genotype. Damaged sperm DNA, abnormal sperm chromatin structure and failure to form a male pronucleus in fertilized embryos have been reported to be linked to retarded embryo development\textsuperscript{68,69}. Our analysis of transposon RNA abundance in pi6 mutant germ cells argues against a role for pi6 piRNAs in transposon silencing during spermatogenesis, but we cannot currently exclude a direct or indirect role for pi6 piRNAs in silencing transposons in the early embryo\textsuperscript{67}. Of course, DNA damage might reflect incomplete repair of the double-stranded DNA breaks required for recombination, rather than transposition or transposon-induced illegitimate recombination.

How pachytene piRNAs identify their targets remains poorly understood in part because of a lack of suitable biochemical or genetic model systems. The availability of a mouse mutant missing a specific set of piRNAs whose absence causes a readily detectable phenotype should provide an additional tool for understanding the base-pairing rules that govern the binding of piRNAs to their RNA targets, and for unraveling the regulatory network created by pachytene piRNA. Finally, we note that in many placental mammals, the syntenic location corresponding to pi6 also produces piRNAs. Yet, despite the importance of pi6 piRNAs for mouse fertility, the actual sequence of the pi6 precursor transcript is conserved only among rodents\textsuperscript{71}. Explaining how the essential function of fertility can rely on regulatory molecules whose sequence is so poorly conserved remains the central challenge of pachytene piRNA biology.

Online content
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Methods

Mouse mutants. Mice were maintained and killed according to guidelines approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School (A-2222-17). Small guide RNA-pairing piRNA constructs were designed using CRISPR design tools (crispr.mit.edu), DNA oligonucleotides containing guide sequences were cloned into pX330 vectors74, and their cleavage activity tested in NIH3T3 cells by cotransfecting pX330 constructs containing sgRNA sequences and porcymycin-resistant plasmid (pPUR) using TransIT-T2 (Mirus Bio). Puromycin (3 µg/ml) was added 24 h after transfection and DNA was extracted 48 h afterwards. Promoter deletions were detected by PCR using primers flanking the predicted Cas9 cleavage sites.

For mice, sgRNA were generated by in vitro transcription and purified by electrophoresis on 8% (w/v) polyacrylamide gels. To generate the pi6em1 and pi7em1 lines used in this study, in vitro-transcribed sgRNAs (10 ng/µl) each targeting pi6 and pi7 were mixed with Cas9 mRNA (40 ng/µl) and injected together into the cytoplasm of one-cell C57BL/6EeJ (zygote only). For some founders, the sgRNA and Cas9 mRNA mixture was combined with pX330 plasmids expressing the same four sgRNAs and Cas9 and injected into the cytoplasm and pronuclei of one-cell C57BL/6EeJ (zygote + DNA). For pi6em8, in vitro-transcribed sgRNAs and Cas9 mRNA were injected into the cytoplasm of one-cell C57BL/6EeJ embryos. Embryos were transferred to pseudopregnant females using standard methods. To screen for mutant founders, DNA was extracted from small pieces of tail clipped from 3-week-old pups. Deletions were detected by PCR, and PCR products were purified and cloned into TOPO blunt vectors. Mutant sequences were determined by Sanger sequencing. Mouse mutant lines were validated by PCR, and PCR products were purified and cloned into TOPO blunt vectors. pi6em8 and pi6em1 mutant founders were backcrossed for at least two generations before use.

Mouse fertility test. Each 2–8 month-old male mouse was housed with one 2–8 month-old C57BL/6EeJ female, who was examined for the presence of a vaginal plug the following morning. When a plug was observed, the female was housed separately. For male mice that did not produce pups for 3 months (~3 cycles), the original female was replaced with a new female and the fertility test continued.

Tests histology, sperm count and sperm morphology. Mouse testes were fixed in Bouin’s solution overnight, washed with 70% ethanol, embedded in paraffin (10 µm sections), deparaffinized and rehydrated in serial dilutions of ethanol, then stained with hematoxylin and eosin. Sections were counterstained with Mayer’s hemalum, dehydrated in ethanol, cleared in xylene, and mounted in DPX. Leica DMi8 fluorescent microscope equipped with a 20× objective with 0.4 numerical aperture (NA) was used to examine sections. PI staining and DAPI nuclear staining was performed as described74. Mouse testes were incubated in hypotonic buffer (30 mM Tris-Cl, pH 8.2, 50 mM sucrose, 17 mM sodium citrate, 5 mM EDTA, 0.5 mM MgCl2, 0.075 mg ml−1 penicillin-G, 0.05 mg ml−1 streptomycin sulfate, 0.02% (v/v) phenol red, 4 mM MgSO4, 250 µg ml−1 Hoechst 33342 (Thermo Fisher Scientific) and rotated at 150 r.p.m. for 15 min. Separated seminiferous tubules were washed with 1X GS containing 0.4 mg ml−1 DNase I and 1 µg ml−1 trypsin and 1µg ml−1 DNase I in 1X GS at 37°C and rotated at 150 r.p.m. for 15 min. Tubules were incubated on ice for 15 min after being washed in cold 1X GS. Testis histology, sperm count and sperm morphology. Mouse testes were fixed in Bouin’s solution overnight, washed with 70% ethanol, embedded in paraffin (10 µm sections), deparaffinized and rehydrated in serial dilutions of ethanol, then stained with hematoxylin and eosin. Leica DMi8 fluorescent microscope equipped with a 20× objective with 0.4 numerical aperture (NA) was used to examine sections. PI staining and DAPI nuclear staining was performed as described74. Mouse testes were incubated in hypotonic buffer (30 mM Tris-Cl, pH 8.2, 50 mM sucrose, 17 mM sodium citrate, 5 mM EDTA, 0.5 mM MgCl2, 0.075 mg ml−1 penicillin-G, 0.05 mg ml−1 streptomycin sulfate, 0.02% (v/v) phenol red, 1 mg ml−1 Hoechst 33342 (Thermo Fisher Scientific) and rotated at 150 r.p.m. at 33°C for 5 min. Propidium iodide (0.2 µg/ml), final concentration; Thermo Fisher Scientific) was added, and cells strained through a pre-wetted 40 µm cell strainer. Sperm sorting was performed on a FACS aria II (BD Biosciences). The purity of sorted fractions was assessed by immunostaining. Secondary spermatocyte and spermatid populations were >90% pure, and the pachytenie spermatocytes and diplotene spermatocytes were >80% pure.

IVF and embryo transfer. IVF was performed as previously described using spermatozoa from caudal epididymis of C57BL/6, pi6em8 or pi6em1 mice. Spermatozoa were incubated in complete human tubal fluid media (HTF, 101.6 mM NaCl, 4.69 mM KCl, 0.37 mM KH2PO4, 0.2 mM MgSO4, 7 hO2, 21.44 mM Na lactate, 0.33 mM Na pyruvate, 2.78 mM glucose, 25 mM NaHCO3, 2.04 mM CaCl2, 2.04 H2O, 0.075 mg ml−1 penicillin-G, 0.05 mg ml−1 streptomycin sulfate, 0.02% (v/v) phenol red, 4 mM MgSO4, 250 µg ml−1 Hoechst 33342 (Thermo Fisher Scientific) and rotated at 150 r.p.m. for 15 min. Separated seminiferous tubules were washed with 1X GS containing 5% (v/v) FBS, 1 µg ml−1 DNase I and 5 µg ml−1 Hoechst 33342 (Thermo Fisher Scientific) and rotated at 150 r.p.m. at 33°C for 35 min. Propidium iodide (0.2 µg/ml), final concentration; Thermo Fisher Scientific) was added, and cells strained through a pre-wetted 40 µm cell strainer. Sperm sorting was performed on a FACSaria II (BD Biosciences). The purity of sorted fractions was assessed by immunostaining. Secondary spermatocyte and spermatid populations were >90% pure, and the pachytenie spermatocytes and diplotene spermatocytes were >80% pure.

Sperm motility. Caudal epididymal spermatozoa were collected from mice and placed in 3% (v/v) BSA in an incubator with 5% CO2 where a drop of sperm was removed from the suspension and pipetted into a sperm counting glass chamber, then assayed by CASA or video acquisition. CASA was conducted using an IVOS II instrument (Hamilton Thorne) with the following settings: 100 frames acquired at 60Hz; minimal contrast, 30; 4-pixel minimal cell size; minimal static contrast, 5; 0% straightness (STR) threshold; 10 µs VAP Cutoff; prog. min VAP 20µm s−1; 10 µm s−1 VSL Cutoff; 5-pixel cell size; cell intensity, 90; static head size, 0.30–2.69; static head intensity, 0.10–1.75; static elongation, 10–94; slow cells motility, yes; ×6.88 magnification; LED illumination intensity, 3,000; IDENT illumination intensity, 5,653; 37°C. The raw data files (that is, .dbt files for motile sperm and .dbd files for static sperm) were used for sperm motility analysis. For the motile sperm, only those whose movement was captured with ≥45 consecutive frames were analyzed. For the box plots, the number of static sperm was recalculated for each mouse according to the percentage of motile sperm with ≥45 frames. For hyperactivated motility analysis, .dbt files of motile sperm were used as input for CASAanov, as previously described78. For movie acquisition, a Nikon DS-5M digital microscope with dark-field illumination set to 10 magnification was used to record sperm movement at 37°C. Ex vivo acrosome reaction and capacitation assay. Ex vivo, the acrosome reaction occurs spontaneously in some sperm and can also be triggered by the Ca2+ ionophore A23187 (ref. 1), which results in an acrosome reaction that is visually indistinguishable from that triggered by natural ligands, such as progesterone79 or

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ZP3 (ref. 15), while bypassing signaling pathways essential for acrosome reaction in vivo14. Sperm capacitation was induced and acrosome reaction was assessed as described 15. Cauda epididymides were collected from mice and placed in HTF media containing 10% FBS, 2% PVA, 5% glucose, 4% BSA, 5% glycerol, 0.1% Na2H in a humidified incubator at 5% CO2. A few incisions were made in the epididymides with scissors to release the sperm, followed by incubation at 37 °C in 5% CO2 for 90 min. Calcium ionophore A23187 (10 μM final concentration in DMSO) was added, and incubation continued for 30 min. Sperm were fixed at room temperature for 10 min by adding two volumes of 4% paraformaldehyde, 0.1% Triton X-100, washed 3 times with 1× PBS, and re-suspended in fresh 1× PBS, spotted on a glass slide and air-dried. Methanol was pipetted onto the sperm to permeabilize the cells, followed by washing with 1× PBS. Slides were incubated overnight in 10 ng·ml−1 Alexa Fluor 488-conjugated peanut agglutinin (PNA) in 1× PBS15, washed with 1× PBS, air-dried and mounted with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Sperm were imaged using a Leica DMi8 fluorescence microscope equipped with a x63, 1.4 NA oil immersion objective (HC PL APO; Leica Microsystems) and analyzed using ImageJ (v2.0.0-r-68/1.52c; https://fiji.sc/).

Transmission electron microscopy. Mouse caudal epididymides were dissected and immediately fixed by immersion in Karnovsky’s fixative (2% formaldehyde (v/v) and 3% glutaraldehyde (v/v) in 0.1 M sodium phosphate buffer, pH 7.4; Electron Microscopy Sciences) overnight at 4 °C, and washed three times in 0.1 M phosphate buffer. Following the third wash, the tissues were postfixied in 1% osmium tetroxide (v/v; Electron Microscopy Sciences) for 1 h at room temperature. Tissues were then dehydrated with 70% to 95% ethanol in 10 min each and embedded using a graded series of 30%, 50%, 70%, 85%, 95%, 100% (three changes) ethanol and 100% propylene oxide (two changes) and a mixture of 50% propylene oxide (v/v) and 50% SPI-Pon 812 resin mixture (v/v; SPI Supplies). The samples were incubated in seven successive changes of SPI-Pon 812 resin over 3 d, polymerized at 68 °C in flat molds and reoriented to allow cross-sectioning of spermatozoa in the lumen of epididymis. Sections measuring 100–150 nm thick were cut on a Leica EM UC7 ultramicrotome (Leica Microsystems) using a diamond knife, collected on copper mesh grids and stained with 3% lead citrate (w/v) and 0.1% uranyl acetate (w/v) to increase contrast. Finally, sections were examined using a Philips CM10 transmission electron microscope (Philips Electronics Optics, Eindhoven, The Netherlands) at 100 kV. Images were recorded using the ERlangschen digital camera system (Gatan).

RNA-seq and small RNA-seq analysis. Small RNA-seq and RNA-seq libraries were constructed incorporating unique molecular identifiers for removal of PCR duplicates and 2% PEG (Inllumina) as described 14,15. To sequence mature piRNAs, small RNA was oxidized with 25 mM NaOAc in 30 mM sodium borate, 30 mM boric acid (pH 8.6; Sigma–Aldrich) at 25 °C for 30 min. RNA was precipitated with ethanol before adapter ligation. A set of nine synthetic 2′-O-methylated RNA oligonucleotides was added to each RNA sample to allow measurement of molecules per cell. Small RNA-seq and RNA-seq reads were mapped to mouse genome assembly mm10 using piPipes 16. For small RNA quantification, sequences of synthetic spike-in oligonucleotides were identified allowing no mismatches and the number of molecules of small RNAs per library was calculated on the basis of the read abundance of the spike-in oligonucleotides. For long transcript quantification, 1 ml of 1.100 dilution of ERIK spike-in mix 1 (Thermo Fisher, catalog no. A4556740, lot no. 904158382) was added to 1 μg of total RNA in the first step. For Fig. 6, Extended Data Fig. 3 and Supplementary Tables 2 and 5, differentially expressed transcripts were determined using DESeq2 (ref. 17). Statistical testing was performed first using the Wald test and the derived P values were adjusted for multiple testing using the Benjamini–Hochberg procedure, as stated in the figure legends or Methods, Mann–Whitney–Wilcoxon two-sided test was used to calculate P values. For Figs. 2d, 6c and 7a, Extended Data Fig. 2c and Supplementary Videos 1–10, representative data (reproducible in ≥3 individual mice) from a single mouse are shown. See also the Nature Research Reporting Summary.

Data availability

All sequencing data are available through the National Center for Biotechnology Information Sequence Read Archive using accession number PRJNA634688. Source data are provided with this paper.

Code availability

The code used for identifying piRNA-directed cleavage sites is available at https://github.com/weng-lab/GTBuster. All other codes used in this study are described in the Methods and Nature Research Reporting Summary. Source data are provided with this paper.

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**Author contributions**

P.-H.W., K.C., Y.F., Z.W. and P.D.Z. conceived and designed the experiments. P.-H.W., K.C., D.M.O., A.A. and C.C. performed the experiments. Y.F., T.Y., I.G. and P.-H.W. analyzed the sequencing data. P.-H.W., Y.F. and P.D.Z. wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

Extended data is available for this paper at https://doi.org/10.1038/s41588-020-0657-7. Supplementary information is available for this paper at https://doi.org/10.1038/s41588-020-0657-7.

Correspondence and requests for materials should be addressed to P.-H.W., Z.W. or P.D.Z.

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Extended Data Fig. 1 | Confirmation of mutant founder genotypes. 

**a** Genotyping of mutant founders by PCR. Mutant founders were generated by injecting sgRNAs and Cas9 mRNAs into C57BL/6 one-cell zygotes, which were transferred to surrogate mothers and screened after birth. Gel images were cropped for clarity (see also Source Data). Genomic sequences of pi6 promoter region in pi6em1 (b) and pi6em2 (c) mouse lines. The presence of both deleted and undeleted PCR products indicate a heterozygous mutant founder that carries just one CRISPR-edited chromosome. 

**d** Genomic sequences of pi17 promoter region in pi17-/- mouse lines. Dashes, genomic sequences deleted by CRISPR; dots, unaltered sequence omitted for clarity.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | pi6\textsuperscript{-/} adult male phenotype. a, Number of litters produced in 6 months by 2–8 month-old males. b, Body and testis weight of 2–4 month-old pi6\textsuperscript{-/} and pi6\textsuperscript{-/-} males. Each dot represents an individual mouse. Vertical lines denote median; boxes report 75\textsuperscript{th} and 25\textsuperscript{th} percentiles; whiskers indicate the maximal and minimal values. c, Representative spermatozoa from C57BL/6 and pi6\textsuperscript{-/} males. d, Representative patterns of meiotic chromosome synapsis in pi6\textsuperscript{-/} pachytene spermatocytes. SYCP1, Synaptonemal complex protein 1; SYCP3, Synaptonemal complex protein 3. e, Quantification of patterns of meiotic chromosome synapsis depicted in (d) from C57BL/6 (n = 4) and pi6\textsuperscript{-/-} (n = 4) males.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Abundance of transposons in pi6<sup>em1/em1</sup> and pi6<sup>em2/em2</sup> germ cells. **a**, Proportions of the whole genome or piRNA sequences composed of repetitive sequences. **b**, Abundance of mature piRNAs from the top five major pachytene piRNA-producing loci in indicated cell types measured by small RNA-seq. Each dot represents the abundance of unique-mapping reads in one C57BL/6 (n=3) or pi6<sup>em1/em1</sup> (n=3) male. Vertical black lines denote median; boxes report 75<sup>th</sup> and 25<sup>th</sup> percentiles; whiskers indicate the maximal and minimal values. **c**, Abundance of transposon-derived RNAs in mouse germ cells. Each dot represents the mean of four (wild-type and pi6<sup>em1/em1</sup>) or three (pi6<sup>em2/em2</sup>) biologically independent RNA-seq experiments. Gray dots indicate change in abundance <2-fold and/or FDR > 0.05 determined by DESeq2 (see also Methods).
Extended Data Fig. 4 | Pregnancy rate of surrogate mothers in IVF and ICSI experiments. Percent of pregnant surrogate mothers in IVF (a) and ICSI (b).
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Transcripts directly cleaved by pi6 and pi10-qC2-545.1 piRNAs. a, Strategy to identify piRNA-directed cleavage sites. b, pi6-dependent cleavage sites in mRNAs or pachytene piRNA precursors from pi10-qC2-545.1 and pi10-qA3-143.1 showing inferred base pairing with the corresponding pi6 piRNA guides. An exemplary piRNA guide is shown where more than one piRNA can direct the same cleavage. c, Cleavage sites in pi6 precursors explained by pi10-qC2-545.1 piRNAs. An exemplary piRNA guide is shown.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Transcriptome changes in \textit{pi6}^{em1/em1} and \textit{pi6}^{em2/em2} cells. \textbf{a}, Expression of mRNAs measured by qRT-PCR using oligo dT\textsubscript{20} to prime cDNA synthesis and PCR primers spanning \textit{pi6} piRNA-directed cleavage sites (gene names in red) or designed to detect full-length RNA (gene names in black). \textit{Pou2f2} mRNA abundance in spermatids was below the limit of detection by qRT-PCR. \textbf{b}, Abundance of piRNA precursors from the top five major pachytene piRNA-producing loci in indicated cell types measured by RNA-seq. For (\textbf{a}) and (\textbf{b}), thick vertical lines denote median, boxes report 75\textsuperscript{th} and 25\textsuperscript{th} percentiles, and whiskers indicate the maximal and minimal values. Each dot represents an individual mouse.
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Sample size

The investigators reached and confirmed all the conclusions in this manuscript using data collected from 3–11 individual, age-matched (2–4 month old mice and two independently generated mutant lines for all major experiments. The sample size was determined by the standard in the field that enables statistical meaningful comparison for the given experiment. We aimed to exceed the standard sample size unless the availability of data was restricted by the mouse phenotype defects (e.g. male fertility defects in pi6 mutants rendered fewer data points than the controls in Fig 2a.)

Data exclusions

No data are excluded.

Replication

We validated the reproducibility of our data using (1) 3–11 individual animals of identical genetic background (C57BL/6) and a similar age (2–4 month) and (2) two mutant mouse lines independently generated and validated by different CRISPR guides. All observations in this study, including the mutant phenotype and molecular changes, are reproducible in both mutant mouse lines.

Randomization

Animals used in this study were grouped based entirely on their genotypes, involving no subjective animal allocation.

Blinding

The investigators are blinded in experiments for which the order of data collection does not affect the outcome, such as measuring sperm motility and fertilization capability (i.e. in vitro fertilization and intracytoplasmic injection). For sequencing experiments, blinding is not ideal or relevant. Libraries from wild-type and mutant animals are sequenced in the same run when possible to minimize artificial differences created by separate sequencing runs, which requires prior knowledge of the sample identities.

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- Clinical data

Methods

n/a
- Involved in the study

- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging
### Antibodies

**Antibodies used**

For meiotic chromosome immunofluorescence:
- Rabbit polyclonal anti-SCP1 antibody: Abcam Cat# ab15087; Lot# gr215384-18; dilution 1:1000
- Mouse monoclonal anti-SCP3 antibody: Abcam Cat# ab97672; Lot# gr255063-1; dilution 1:1000

**Validation**

Anti-SCP1 antibodies for mouse SYCP1 proteins (ab15087):
Data for validation of the antibodies specificity for immunofluorescence was reported on the manufacturer’s (Abcam) website: https://www.abcam.com/scp1-antibody-ab15087.html. In our hands, the immunostaining pattern using the antibodies (Supplementary Fig. 2d) is consistent with that from the manufacturer and 20 other publications.

Anti-SCP3 antibodies for mouse SYCP3 proteins (ab97672):
Data for validation of the antibodies specificity for immunofluorescence was reported on the manufacturer’s (Abcam) website: https://www.abcam.com/scp3-antibody-cor-10q117-ab97672.html. In our hands, the immunostaining pattern using the antibodies (Supplementary Fig. 2d) is consistent with that from the manufacturer and 60 other publications.

### Eukaryotic cell lines

**Cell line source(s)**

NIH/3T3 was purchased from ATCC: https://www.atcc.org/Products/All/CRL-1658.aspx

**Authentication**

The cell line was tested and authenticated by ATCC using morphology, karyotyping, and PCR.

**Mycoplasma contamination**

The cell line was tested negative for mycoplasma by ATCC using cultures, DNA staining, and PCR.

**Commonly misidentified lines**

No commonly misidentified cell line was used in this study.

### Animals and other organisms

**Laboratory animals**

- C57BL/6J mice: JAX# 000664; wild-type control for all experiments; 2–4 month old males
- pi6em1 mice: generated in the C57BL/6J background in this study; 2–4 month old males
- pi6em2 mice: generated in the C57BL/6J background in this study; 2–4 month old males
- p17+/− mice: generated in the C57BL/6J background in this study; 2–4 month old males
- B6D2F1/J mice: JAX# 100006; oocyte donors for intracytoplasmic injection experiments; 2–4 month old females
- B6SJLF1/J mice: JAX# 100012; oocyte donors for in vitro fertilization experiments; 2–4 month old females
- Swiss Webster mice: Taconic; surrogate mothers for embryo transfer; 2–4 month old females

**Wild animals**

No wild animals were used in this study.

**Field-collected samples**

No field-collected samples were used in this study.

**Ethics oversight**

The Umass Medical School (UMMS) Institutional Animal Care and Use Committee (IACUC) approved the animal procedures under protocol D16-00196 on 16 February 2017.

Note that full information on the approval of the study protocol must also be provided in the manuscript.