pollinated by flower visitors with large foraging ranges, which are usually generalist species, such as honey bees (*12*). In accordance with this hypothesis, we found greater dominance of *Apis* spp. in larger holdings regardless of species richness (fig. S3), and that flower-visitor density effects were enhanced when richness increased in large fields (Fig. 2). Such synergistic influences among pollinator species on crop yield (kg ha^{-1}) are likely due to different nonexclusive mechanisms (*22*), including pollination niche complementarity (*23*, *24*), interspecific interactions (*25*, *26*), or raising the chances of providing effective pollinator species (i.e., sampling effects of biodiversity) (*27*, *28*).

Pollinator deficits have been neglected from previous global or continental yield gap analyses (*5*, *7*, *9*, *10*). However, here we found that they are responsible to a large degree for yield gaps of pollinator-dependent crops in small holdings (Fig. 1 and table S2), even after considering several environmental and management predictors of crop yield (Fig. 3). Indeed, flower-visitor density was the most important predictor of crop yield. Closing flower-visitor density gaps is a realistic objective, as our figures are based on the densities observed in real-world farms (i.e., the difference between the 90th and 10th percentiles). Unfortunately, recent studies suggest that flower-visitor assemblages in agroecosystems are increasingly threatened because of declining floral abundance and diversity, as well as increasing exposure to pesticides and parasites (*15*, *16*). Such trends can be reversed by a combination of practices, the effectiveness of which is context dependent, including sowing flower strips and planting hedgerows, providing nesting resources, more targeted use of pesticides, and/or restoration of seminatural and natural areas adjacent to crops (table S1) (*13*, *29*).

Enhancing smallholder livelihoods through greater crop yields while reducing negative environmental impacts from agriculture is one of the greatest challenges for humanity (*3*, *5*). Moreover, from a food-security point of view, pollinator-dependent crops provide essential micronutrients to human health where needed (*4*). Our data indicate that the effectiveness of ecological intensification through pollination services was greater for small, rather than large, holdings. Using pollination services as a case study, we demonstrated that ecological intensification can create mutually beneficial scenarios between biodiversity and crop yields worldwide.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/351/6271/388/suppl/DC1
Materials and Methods
Figs. S1 to S3
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References (*30*–*44*)
Database S1

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SMALL RNAS

Biogenesis and function of tRNA fragments during sperm maturation and fertilization in mammals

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Several recent studies link parental environments to phenotypes in subsequent generations. In this work, we investigate the mechanism by which paternal diet affects offspring metabolism. Protein restriction in mice affects small RNA (sRNA) levels in mature sperm, with decreased let-7 levels and increased amounts of 5′ fragments of glycine transfer RNAs (tRNAs). In testicular sperm, tRNA fragments are scarce but increase in abundance as sperm mature in the epididymis. Epididymosomes (vesicles that fuse with sperm during epididymal transit) carry RNA payloads matching those of mature sperm and can deliver RNAs to immature sperm in vitro. Functionally, tRNA-glycine-GCC fragments repress genes associated with the endogenous retroelement MERVL, in both embryonic stem cells and embryos. Our results shed light on sRNA biogenesis and its dietary regulation during posttesticular sperm maturation, and they also link tRNA fragments to regulation of endogenous retroelements active in the preimplantation embryo.

Accumulating evidence indicates that parental environments can affect the health of offspring. For example, paternal nutrition influences offspring metabolism in mammals (*1*). Our prior published work showed that male mice consuming a low-protein diet fathered offspring exhibiting altered hepatic cholesterol biosynthesis, relative to the offspring of control males (*2*). The mechanisms by which paternal conditions reprogram offspring phenotype remain elusive, as males can influence offspring via the sperm epigenome, microbiome

transfer, seminal fluid signaling, or indirectly through maternal judgment of mate quality (*3*, *4*). Therefore, we first tested whether metabolic gene expression was altered in offspring generated via in vitro fertilization (IVF) using sperm obtained from animals consuming a control or low-protein diet (19 or 10% protein, respectively) (see supplementary materials and methods). Despite the potential for IVF and embryo culture to obscure paternal effects on offspring metabolism, we found that, compared with control IVF offspring, IVF-derived offspring of males consuming a low-protein diet exhibited significant hepatic up-regulation of the gene encoding the cholesterol biosynthesis enzyme squalene epoxidase (*2*) (fig. S1). This finding demonstrates that paternal diet can affect offspring metabolism via information located in sperm.

Because small RNAs (sRNAs) are central to a broad range of epigenetic phenomena (*5*), we isolated cauda sperm from males consuming a control or low-protein diet and purified small [<40 nucleotide (nt)] RNAs for analysis by deep sequencing. The resultant sequencing libraries reveal markedly abundant (~80% of sRNAs) ~28- to 34-nt tRNA fragments (tRFs), which are predominantly derived from the 5′ ends of tRNAs (Fig. 1, A to D, and tables S1 and S2) (*6*). 5′ tRFs are also abundant in cauda sperm from the bull *Bos taurus* (table S3), which suggests that tRNA cleavage in gametes is conserved among mammals, and perhaps more broadly (*7*). Given the low RNA content of sperm relative to oocytes, we focused our analyses on highly abundant sRNAs in sperm. The low-protein diet affected levels of multiple sRNAs, including highly abundant tRNA fragments, across eight pairs of sperm samples (Fig. 1, E and F). Most notably, 5′ fragments of tRNA-Gly-CCC, -TCC, and -GCC exhibited a ~two- to threefold increase in low-protein sperm, and tRF-Lys-CTT and tRF-His-GTG were similarly up-regulated. In addition to tRFs, other RNA species differ in abundance between sperm samples, with several let-7 species being down-regulated in low-protein sperm.

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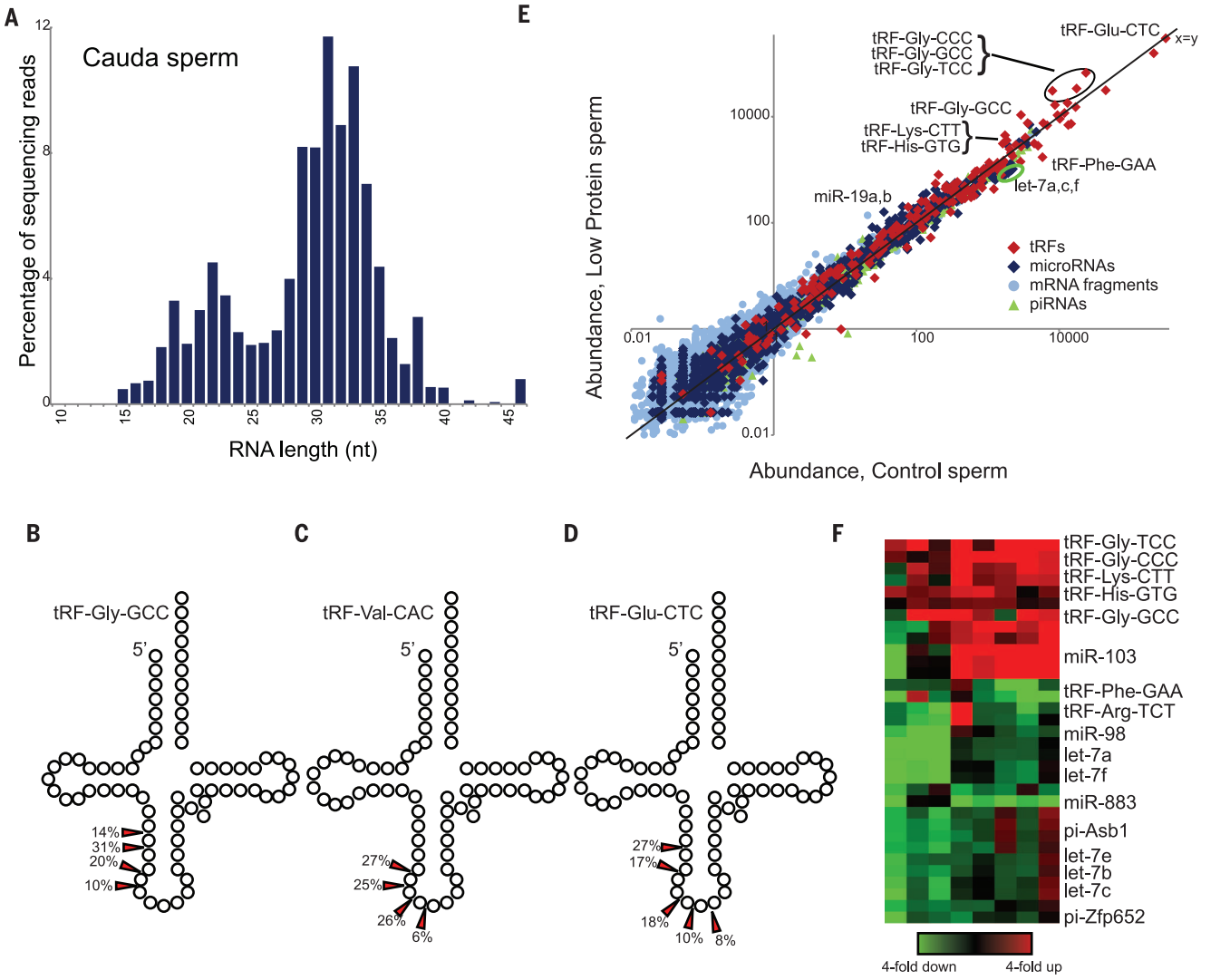

Fig. 1. Dietary effects on sRNAs in sperm. (**A**) Size distribution of sequencing reads for cauda sperm sRNAs. (**B** to **D**) 5′ fragments of tRNA are shown schematically, with arrowheads indicating dominant 3′ ends. (**E**) Dietary effects on sperm sRNA content. The scatter plot shows RNA abundance (in parts per million) for sperm isolated from control animals (*x* axis, $\log_{10}$) versus low-protein sperm (*y* axis), with various RNA classes indicated. Multiple points for tRFs result from sequence differences between genes encoding a given tRNA isoacceptor. (**F**) Heat map showing RNAs responding to diet across eight paired sperm samples.

We next assayed levels of intact tRNAs in the testis, finding no correlation between dietary effects on testicular tRNA levels and tRF changes in cauda sperm (fig. S2). This finding provides evidence against the hypothesis that tRFs in mature sperm result simply from random degradation of tRNAs used during spermatogenesis. Moreover, deep sequencing and Northern blot analyses (Fig. 2, A and C; fig. S3; and tables S1 and S2) revealed very low levels of tRNA fragments in the testes or in various purified testicular spermatocyte/spermatid populations, raising the question of when sperm gain tRFs during maturation. After exiting the testis, sperm continue to mature for several days in the epididymis, and we noted robust tRNA cleavage throughout this tissue (Fig. 2, B and D, and fig. S4). Not only do overall tRF levels increase distally in the male reproductive system, but the spectrum of specific tRFs also differs between the testis, proximal caput epididymis, and distal cauda epididymis (Fig. 2D and table S2).

Because our data suggest that sRNAs in mature sperm could have originated at multiple locations throughout the reproductive tract, we assessed the effect of paternal diet on sRNAs in the testis (n = 9 pairs), caput epididymis (n = 6), and cauda epididymis (n = 5) (fig. S5). Two prominent dietary effects on the cauda sperm RNA repertoire—increased abundance of glycine tRFs and decreased abundance of let-7—were recapitulated in the testis and epididymis but not in the liver, muscle, or blood (table S1). Thus, tissues throughout the male reproductive tract, including mature sperm, exhibit consistent changes in glycine tRFs and let-7 in response to the low-protein diet, suggesting that similar diet-responsive pathways are present throughout the tract and providing technical replication of the fundamental epigenomic changes wrought by the low-protein diet.

Our finding of robust tRNA cleavage in the epididymis but not the testis raises the possibility that the abundant tRFs in the cauda sperm might be trafficked to sperm from the epididymal epithelium, rather than arising during testicular spermatogenesis. During transit through the epididymis, sperm fuse with small extracellular vesicles known as epididymosomes (*8*–*11*). To test the hypothesis that epididymosomes deliver sRNAs (*12*, *13*) to sperm, we purified epididymosomes (fig. S6) and characterized their sRNA payload by deep sequencing. Epididymosomes carry high levels (~87% of reads) of 5′ tRFs such as tRF-Glu-CTC and tRF-Gly-GCC, and sRNAs found in purified epididymosomes closely mirror (correlation coefficient r = 0.96) those in cauda sperm (Fig. 2E and fig. S6). Epididymosomal RNAs were resistant to ribonuclease treatment and were found in epididymosomes from spermless *Tdrd1*$^{-/-}$ mice,

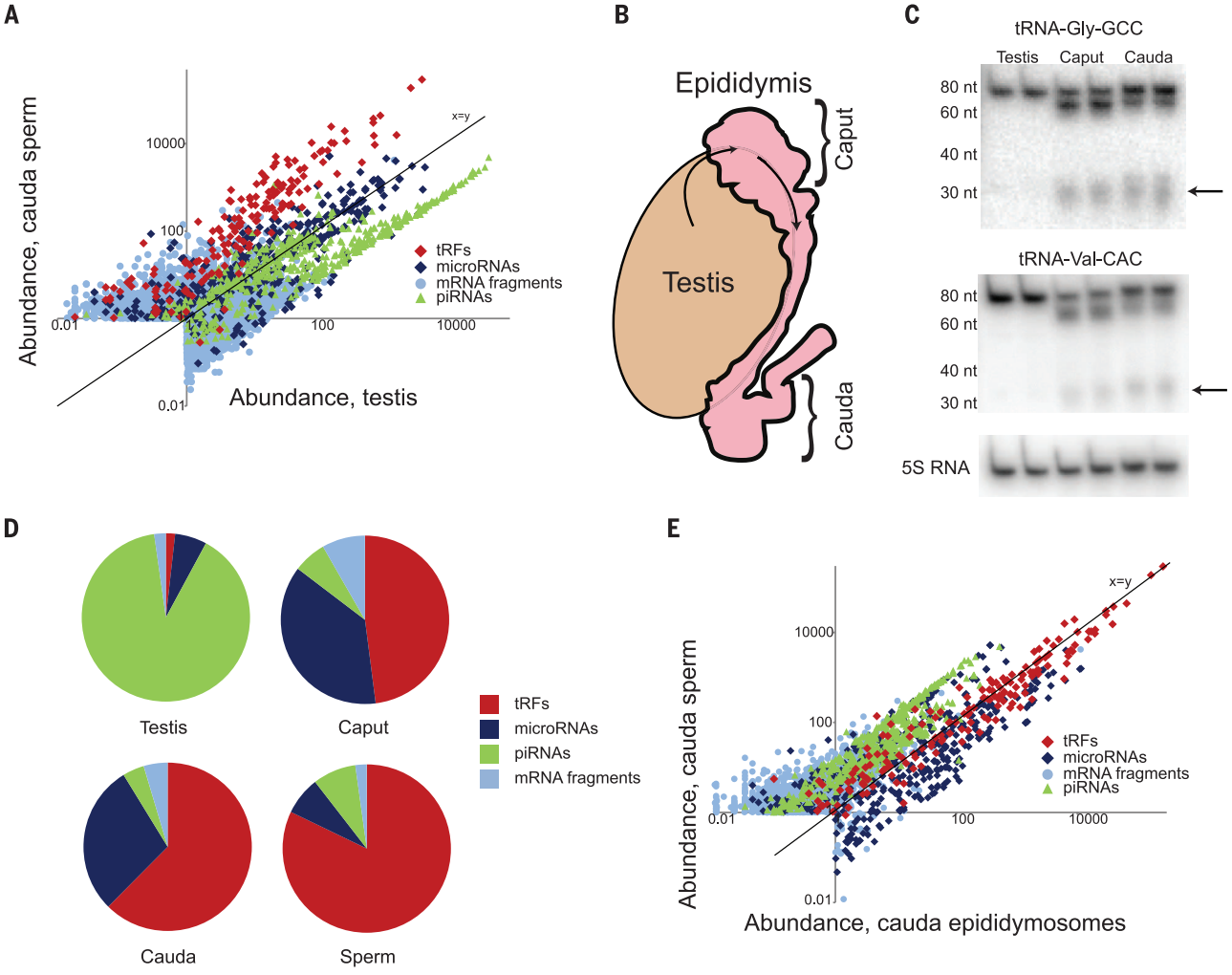

Fig. 2. Cleavage of tRNA occurs predominantly in the epididymis. (**A**) Sperm RNA payload diverges dramatically from testicular RNA. The scatter plot shows sRNAs in the testis versus sperm, as in Fig. 1E. (**B**) Schematic of the epididymis. Sperm exiting the testis enter the proximal (caput) epididymis, then proceed distally to the corpus and cauda epididymis, and exit via the vas deferens. (**C**) tRFs are primarily generated in the epididymis. Northern blots are shown for 5′ ends of tRNA-Gly-GCC or tRNA-Val-CAC on RNA extracted from the testis, caput epididymis, and cauda epididymis. Arrows indicate ~30- to 34-nt 5′ tRFs. 5S RNA served as a loading control. (**D**) Pie charts showing the percentage of sRNAs mapping to the indicated features. (**E**) Scatter plot of sRNA abundance for sperm versus epididymosomes. Sperm-enriched RNAs include piRNAs and fragments of mRNAs involved in spermatogenesis (e.g., *Prm1*) and represent RNAs synthesized during testicular spermatogenesis.

ensuring that vesicles purified from the epididymis are not generated from maturing sperm (fig. S6G).

To further test the hypothesis that epididymosomes are responsible for shaping the RNA payload of sperm, we characterized sRNAs in sperm isolated from the proximal caput epididymis, finding that the RNA payload of caput sperm differs substantially from that of distal cauda sperm (Fig. 3 and fig. S7) (*14*). Proximal-distal biases for specific tRFs along the epididymis were reflected in maturing sperm, showing a dramatic ~10-fold enrichment of tRF-Val-CAC, for example, in cauda relative to caput samples. To directly test whether epididymosomes can deliver their RNAs to caput sperm, we purified caput sperm and incubated them with cauda epididymosomes, then pelleted and washed the resulting reconstituted sperm. Epididymosomal fusion with caput sperm was sufficient to deliver tRF-Val-CAC and other cauda-enriched tRFs to caput sperm in both mouse and bull (Fig. 3, C and D) (*15*). Taken together, these results are most consistent with a mechanism of RNA biogenesis in mammalian sperm in which tRFs generated in the epididymis are trafficked to sperm in epididymosomes. However, our results do not rule out the alternative hypothesis that intact tRNAs in immature sperm (fig. S7F) are cleaved as sperm mature in the epididymis.

We next turned to potential functions of diet-regulated tRFs, using an embryonic stem (ES) cell system amenable to mechanistic analysis. We used antisense locked nucleic acid (LNA)–containing oligonucleotides to interfere with the function of specific tRFs and assayed mRNA abundance as a readout of tRF inhibition. Most antisense oligos had no effect on mRNA abundance (table S4), suggesting that the targeted tRFs are not functional in ES cells or exert regulatory effects that are not assayed by mRNA abundance (*16*). In contrast, interfering with tRF-Gly-GCC resulted in dramatic up-regulation of ~70 genes (Fig. 4, A to C). These genes were unaffected by antisense oligos against other tRNA-Gly isoacceptors or against the middle or 3′ end of tRNA-Gly-GCC (Fig. 4B). The genes derepressed upon tRF-Gly-GCC inhibition are highly expressed in preimplantation embryos and are regulated by the long terminal repeat (LTR) of the endogenous retroelement MERVL (*17*) (Fig. 4D). Regulation of MERVL LTR-driven transcription by tRF-Gly-GCC could be recapitulated in LTR reporter cell lines and is not secondary to translational effects of tRF-Gly-GCC inhibition (figs. S8 and S9 and table S5). Microinjection of antisense oligos targeting tRF-Gly-GCC into zygotes also resulted in derepression of MERVL targets later in development (Fig. 4, E and F, and table S6), indicating that tRF-Gly-GCC also regulates MERVL in a more physiological context.

Given the robust connection between a diet-regulated sRNA and a highly specific set of target genes, we asked whether tRF-Gly-GCC targets are affected in preimplantation embryos generated using sperm from animals consuming a control or low-protein diet. RNA sequencing (RNA-seq) (*18*, *19*) of individual mouse embryos cultured to various stages of development robustly clustered embryos by developmental stage (Fig. 5, A and B; fig. S10; and table S7), with the first two principal components of the data set representing oocyte-derived transcripts and the products of embryonic genome activation.

Because single-embryo RNA-seq data are not suitable for identification of modest changes in individual mRNAs, we searched for consistent changes in larger gene sets: the subset of MERVL targets that respond to tRF-Gly-GCC inhibition (Fig. 4) and the remaining MERVL targets (*17*). At the two-cell stage, both tRF-Gly-GCC targets and the remaining MERVL targets were down-regulated in low-protein embryos relative to controls (Fig. 5C), consistent with the hypothesis that tRF-Gly-GCC in sperm affects expression of MERVL targets in early embryos. We carried out several independent tests of this hypothesis. First, we injected <40-nt RNA populations purified from control and low-protein sperm into control zygotes and discovered that low-protein RNAs could inhibit tRF-Gly-GCC targets in two-cell embryos (Fig. 5D and fig. S11A), indicating that paternal diet can affect preimplantation gene regulation via RNAs in sperm. Second, further defining the relevant RNA from low-protein sperm, microinjection of a synthetic tRF-Gly-GCC oligo resulted in repression of MERVL target genes in two-cell embryos (Fig. 5E and fig. S11B). In addition, because tRFs in sperm are gained during epididymal

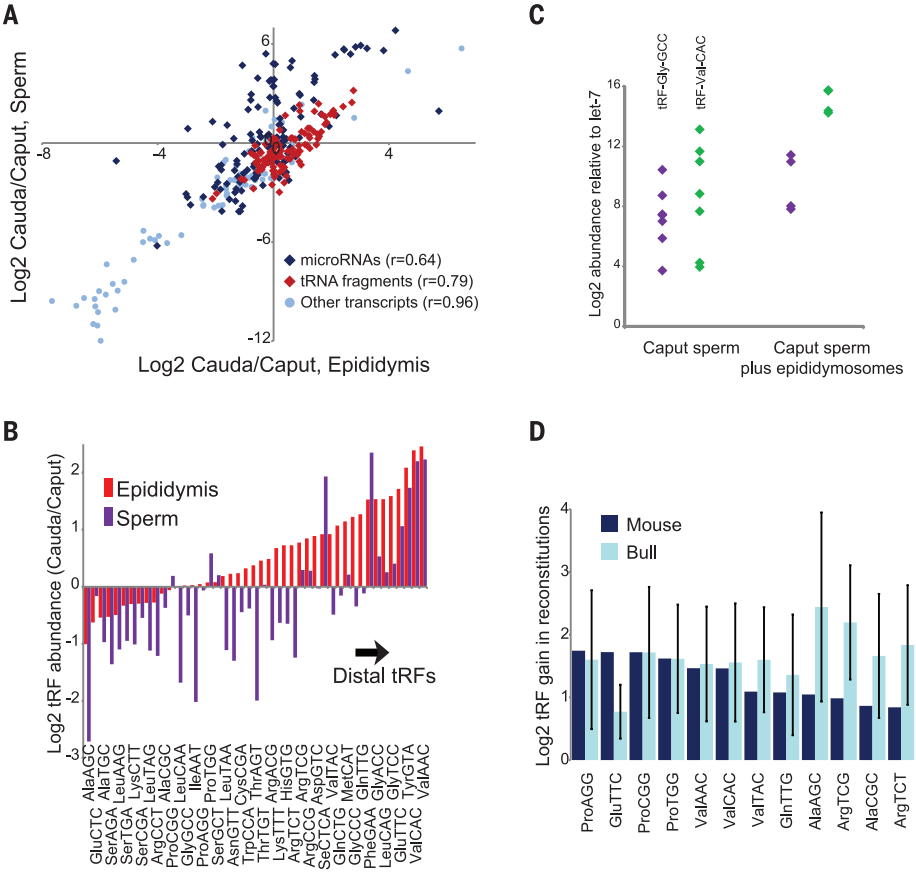

Fig. 3. Changes in sperm tRF payload during epididymal transit. (**A**) Proximal-distal biases observed for RNAs in the epididymis are recapitulated in sperm samples. (**B**) Proximal-distal biases for tRFs, aggregated by anticodon, in the epididymis and sperm. (**C**) TaqMan assay of the indicated tRFs in caput sperm and reconstituted sperm, showing gain of tRFs relative to let-7 (*t* test, P = 0.05 for Gly-GCC and 0.004 for Val-CAC). (**D**) Deep sequencing of reconstituted sperm. tRFs are aggregated by codon and normalized to levels of tRF-Glu-CTC. Caput versus cauda differences were broadly recapitulated in reconstitutions, with tRFs such as tRF-Val-CAC being delivered to caput sperm via fusion with the cauda epididymosomes.

transit (fig. S3), we used testicular spermatozoa or cauda sperm to generate embryos via intracytoplasmic sperm injection (ICSI). Consistent with the higher levels of tRF-Gly-GCC in cauda sperm, embryos generated using cauda sperm expressed MERVL targets at lower levels than embryos generated using testicular sperm (Fig. 5F and fig. S12). Together, these findings all support the hypothesis that tRF-Gly-GCC in sperm is capable of delaying or repressing MERVL target expression in two-cell embryos.

Finally, we note that tRF-Gly-GCC is one of several abundant RNAs regulated by a low-protein diet, and MERVL-driven genes are not the only diet-responsive genes in preimplantation embryos. Ribosomal protein genes were down-regulated in low-protein embryos, and correspondingly, low-protein embryos developed slower than controls (fig. S13) (*20*). Whether altered preimplantation growth kinetics or regulation of MERVL targets could be responsible for the eventual metabolic consequences in offspring remains to be determined. However, as the MERVL program is linked to totipotency (*17*), we speculate that tRF-Gly-GCC repression of MERVL-regulated genes could affect placental size or function, causing downstream effects on metabolism secondary to altered placentation (*1*).

Taken together, our data identify a role for paternal diet in regulating the sperm epigenome in mammals. We show that (i) paternal diet can influence offspring phenotype via information in sperm; (ii) diet alters the level of sRNAs, including tRNA fragments, throughout the male reproductive tract and in mature sperm; and (iii) tRNA fragments can regulate expression of transcripts driven by endogenous retroelements. Our data also illuminate temporal dynamics of sRNA biogenesis during posttesticular sperm maturation and suggest that epididymosomes traffic RNAs from somatic cells of the epididymis to maturing gametes.

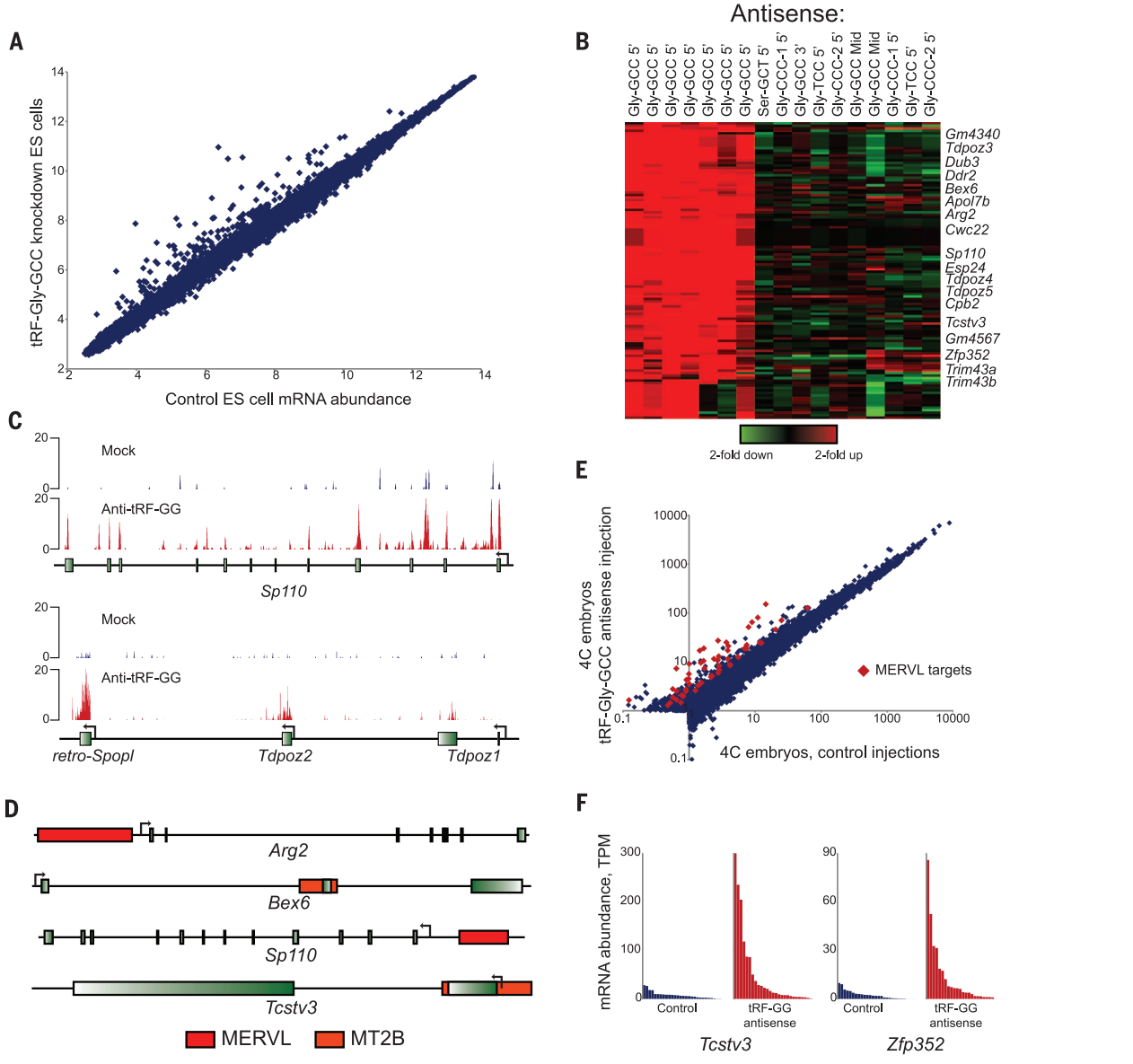

Fig. 4. Regulation of MERVL-driven transcripts by tRF-Gly-GCC. (**A**) Scatter plot shows mRNA abundance in ES cells transfected with green fluorescent protein (GFP) small interfering RNA (siRNA), as compared with LNA antisense oligonucleotides to the 5′ end of tRF-Gly-GCC. (**B**) Effect of tRF-Gly-GCC inhibition on MERVL-regulated genes is isoacceptor-specific. Affymetrix data for ES cells transfected with LNA antisense oligos, relative to matched GFP controls, showing genes changing twofold in two or more samples. (**C**) RNA-seq data for ESCs transfected with GFP siRNA or anti-tRF-Gly-GCC. (**D**) Genomic context of tRF-Gly-GCC target genes, showing nearby MERVL LTRs. (**E**) Inhibition of tRF-Gly-GCC affects MERVL targets in embryos. Averaged single-embryo RNA-seq data for control (n = 28) or tRF-inhibited (n = 27) four-cell-stage (4C) embryos. Among twofold up-regulated genes, known MERVL targets (*17*) are indicated. (**F**) Single-embryo data for two MERVL targets. TPM, transcripts per million.

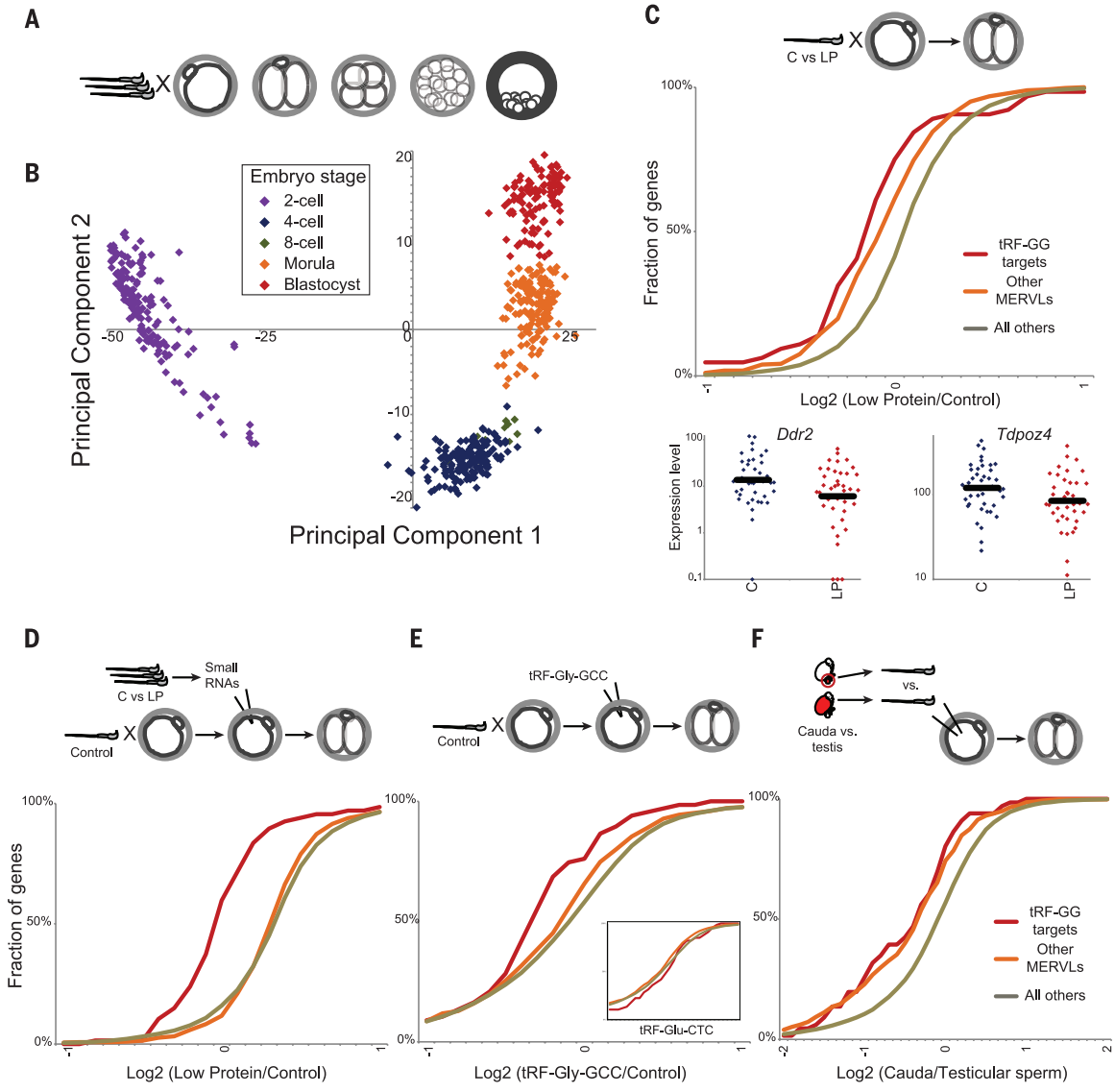

Fig. 5. Paternal dietary effects on preimplantation development. (**A**) Embryos generated by IVF were cultured for varying times and then subjected to single-embryo RNA-seq. (**B**) Single-embryo data for preimplantation embryos represented via principal components analysis: The first two principal components explain 74% of the data set variance. (**C**) Abundance of mRNA in two-cell embryos generated via IVF using control versus low-protein sperm (n = 41 C and 39 LP embryos). Cumulative distribution plots for tRF-Gly-GCC targets ($P = 4.5 \times 10^{-7}$, Kolmogorov-Smirnov test), other MERVL targets (*17*) ($P = 2.5 \times 10^{-13}$), and all remaining genes, showing the percentage of genes with the average $\log_2$(LP/C) indicated on the x axis. Low-protein embryos exhibit a significant shift to lower expression of MERVL targets. Bottom panels show individual embryo data for two targets. (**D**) Small RNAs isolated from control or low-protein cauda sperm were microinjected into control zygotes. RNA-seq (n = 42 C and 46 LP embryos) reveals down-regulation of tRF-Gly-GCC targets ($P = 4.8 \times 10^{-14}$) driven by low-protein RNAs. (**E**) Effects of synthetic tRF-Gly-GCC on two-cell gene regulation, showing significant (P = 0.0001) down-regulation of target genes in embryos injected with tRF-Gly-GCC (n = 26) versus GFP controls (n = 11). The inset shows effects of tRF-Glu-CTC (n = 6). (**F**) Effects of epididymal passage on embryonic gene regulation. Intact sperm isolated from the rete testis (n = 12) or cauda epididymis (n = 9) were injected into control oocytes, and mRNA abundance was analyzed as described above.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/351/6271/391/suppl/DC1
Materials and Methods
Figs. S1 to S13
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References (*21*–*35*)

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Biogenesis and function of tRNA fragments during sperm maturation and fertilization in mammals

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