

RESEARCH PAPER

Deciphering RNA modification dynamics during spermatogenesis and sperm maturation

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Emerging evidence highlights the pivotal roles of RNA modifications in affecting male fertility and offspring health. However, the dynamic landscapes of RNA modification during spermatogenesis and sperm maturation are still incompletely characterized. Here, we profiled 27 RNA modifications across total RNA, tRNA-enriched fractions, and small RNA fractions in developing mouse testes (P7–P56), isolated spermatogenic cells, and epididymal sperm by applying a liquid chromatography-tandem mass spectrometry-based RNA modification quantification platform, deciphered the developmental dynamics of RNA modification signature and coordinative inter-modification cross-talk. Through integrative analyses of RNA modification signatures among spermatogenic cells in the testis and spermatids at different maturation stages in the epididymis, we found that most RNA modifications were gradually reprogrammed from the testis to the epididymis, and finally declined in mature sperm. Specifically, some RNA modifications (e.g., m⁶A and m⁵U) were enriched in meiotic prophase II spermatocytes and round spermatids, while others (e.g., m⁵C and m²G) exhibited high abundance in caput sperm. Additionally, using a type 2 diabetes mellitus mouse model, we found that sperm RNA modification signatures are dynamically reprogrammed during epididymal maturation in response to diabetes, leading to a remarkable alteration in sperm RNA modification signatures and inter-modification correlation networks. Collectively, our study delineates the dynamic landscape of RNA modifications during spermatogenesis and maturation, uncovering its origins and trajectory alterations in the type 2 diabetes mellitus mouse model. This provides novel insights into both the underpinned regulatory mechanisms of RNA modification-dependent spermatogenesis and epigenetic remodeling of sperm during epididymal maturation.

RNA modification | spermatogenesis | epididymis | T2DM (type 2 diabetes mellitus) | epigenetic

INTRODUCTION

Spermatogenesis in mammals involves the mitotic proliferation of spermatogonia, meiotic division of spermatocytes, and spermiogenesis of haploid spermatids (Griswold, 2016). In mice, spermatogenesis follows a defined developmental timeline after birth. Testes from postnatal day 1 (P1) mice primarily contain spermatogonial stem cells (SSCs), which undergo mitotic proliferation for approximately 7 d before differentiating into spermatocytes. Meiosis completes around P21, producing haploid round spermatids. These then undergo a 7 d spermiogenesis process involving nuclear compaction, cytoplasmic extrusion, and flagellum formation, ultimately forming elongated spermatids by P35 (Griswold, 2016). After the first round of spermatogenesis completes in the testes, the newly generated sperm subsequently enter the epididymis—a long, coiled tubular organ attached to the testis—for further maturation (Johnston et

al., 2005). Anatomically, the epididymis comprises three segments: the caput (head), corpus (body), and cauda (tail) (Belleannée et al., 2012). During 10–14 d of epididymal transit, spermatozoa acquire fertilization competence. Mature, functional sperm are then stored in the cauda epididymidis and vas deferens (Gervasi and Visconti, 2017; Zhou et al., 2025). However, how this sophisticated sequential biological process is orchestrated remains poorly understood.

Studies have reported that epigenetic regulation is actively involved in mammalian spermatogenesis, including DNA methylation (Dura et al., 2022), histone modifications (Lin et al., 2025; Qian et al., 2013), RNA modifications, and non-coding RNAs (Niu et al., 2011; Wang et al., 2023b). Among them, RNA modifications, such as m⁶A and m⁵C, have garnered significant attention due to their pivotal roles in post-transcriptional regulation across various biological processes and diseases (Barbieri and Kouzarides, 2020; Tang et al., 2018; Wu et al.,

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2022; Xiong et al., 2025; Yang et al., 2022; Yang et al., 2023; Yao et al., 2023; Yoon et al., 2017). Specifically, dysregulation of the modifiers of m⁶A (Lin et al., 2017; Xu et al., 2017), m⁵C (Blanco et al., 2011), or ac⁴C (Chen et al., 2022b; Zhu et al., 2025) leads to male infertility featured with impaired spermatogonia maintenance and differentiation (Lin et al., 2017) or meiotic arrest (Chen et al., 2022b). Moreover, sperm RNA modifications also exhibit regulatory capacity in intergenerational epigenetic inheritance. Deletion of Dnmt2, a tRNA m⁵C methyltransferase, inhibits the upregulation of m⁵C level on sperm small RNA upon high-fat diet exposure, and abolishes the capability of sperm RNA in transmitting paternally acquired metabolic disorder to the offspring (Zhang et al., 2018). This evidence indicates that sperm RNA modification dynamics, together with sperm RNA profile diversity, generate a unique “sperm RNA code” signature upon specific environmental exposure (He et al., 2021). Sperm RNA code refers to the abundances of a diverse repertoire of RNAs stored in sperm, along with their specific modifications’ abundances, serving as paternal epigenetic information carriers to mediate intergenerational inheritance of acquired traits (Chen et al., 2016a; Wang et al., 2022; Zhang et al., 2019; Zhang et al., 2018). However, where and how sperm gain this “sperm RNA code” signature are still debatable (Zhang et al., 2019). Emerging evidence showed that sperm acquire considerable small RNAs from the epididymis during maturation, such as tsRNA and microRNA (Conine et al., 2018; Frye et al., 2018; Sharma et al., 2018), while some researchers hold the idea that the small RNA composition signatures in epididymis mature sperm are more like those in testis sperm (Wang et al., 2023a). As to RNA modifications, beyond these well-studied RNA modifications (e.g., m⁶A, m⁵C), there are over 170 types of RNA modifications that have been identified (Boccalletto et al., 2022), of which the regulatory roles in spermatogenesis and sperm maturation remain largely unexplored, and a comprehensive, time-resolved RNA modification landscape spanning testicular development with the entire spermatogenic cycle has yet to be elucidated.

In this study, we have collected testicular tissues at different developmental stages, purified key spermatogenic cell populations from adult mice testes, isolated sperm from distinct epididymal segments, and used a liquid chromatography-tandem mass spectrometry-based (LC-MS/MS) RNA modification quantification platform to systematically decipher the RNA modification landscape across testicular development and epididymal maturation. We also established a type 2 diabetes mellitus (T2DM) mouse model for the comparative analyses of sperm RNA modification alteration signatures during epididymal maturation. Collectively, our study generated a dynamic, high-resolution RNA modification map throughout testicular development and sperm maturation, and demonstrated the stage-specific reorganization of inter-modification cross-talk dynamics during these biological processes, suggesting an active regulation of RNA modification in mediating male fertility and intergenerational epigenetic inheritance.

RESULTS

Various RNA modifications show dynamic changes during testicular development.

Male germ cells, such as spermatogonia, spermatocytes in

meiosis prophase I, II, round spermatids, elongating spermatids, and sperm, are produced sequentially during testes development after birth. To profile the RNA modification dynamics in testes across postnatal days, we quantified 27 types of RNA modification in total RNA, tRNA-enriched fractions (~80 nt), and small RNA fractions (15–50 nt) by an LC-MS/MS based RNA modification detection platform across testes from mice at P7, P14, P21, P28, P56 (Figure 1A and B). Overall modification rate analyses of adenine (A), uracil (U), cytosine (C), and guanine (G) bases revealed that tRNA-enriched fractions possess a higher modification rate than total RNA and small RNA fractions across different developmental stages, particularly on C/G/U bases (Figure S1A). As for A bases, the modification rates were comparable in total RNAs and tRNA-enriched fractions (Figure S1A). Notably, the overall modification rate on tRNA fraction significantly increased along with postnatal days (except for U-base modifications) (Figure S1A). Hierarchical clustering analyses further highlight this alteration trend in tRNA-enriched fractions (Figure S1B): most modifications (e.g., m⁵C, m¹I, m¹A, m⁷G) show the lowest abundance at P7, then gradually increase, and peak at P56 (Figure 1C; Figure S1B). As for total RNA and small RNA fractions, distinct modification alteration trends were observed. In total RNA, most modification abundances are increased along with postnatal days at first, and reach a peak at P28, then either maintain the high level (e.g., m¹I, m²G, m¹G) or decrease (e.g., Ψ, Am, Gm) at P56 (Figure S1B). While most small RNA fractions’ modifications showed the highest enrichment at P7 (e.g., m²₂G, m³C) (Figure S1B), the modification level fluctuates afterwards. Given the established role of NAT10 in ac⁴C formation, studies have shown that NAT10 protein levels begin to rise from P3, peak at P28, and subsequently decline (Chen et al., 2022b), which is fully consistent with the developmental changes in ac⁴C modification in total RNA that we detected (Figure S1B). To further clearly depict the temporal dynamics of specific RNA modifications, we quantified m²G, m¹G, Gm, Cm, in total RNA; m⁷G, m⁵C, m¹A, m¹G in tRNA-enriched fractions; Gm, Am, m⁵C, m¹G in small RNA fractions across postnatal testis development (Figure 1D). In tRNA-enriched fractions, m⁵C and m¹G abundance exhibited progressive increases along with postnatal days (Figure 1D). In contrast, total RNA and small RNA fractions modifications displayed more diverse patterns: within total RNA, m¹G and m²G steadily increased across postnatal days, while Gm and Cm showed biphasic patterns peaking at P28 and then declining (Figure 1D).

Reshaping of testes RNA modification landscape and inter-modification cross-talk along spermatogenesis

To further evaluate the stage-specific RNA modification signature across testicular development, we next performed principal component analysis (PCA) to distinguish developmental stages based on the RNA modification signatures of total RNA, tRNA-enriched and small RNA fractions (Figure 1E; Figure S1C–E). The PCA plot revealed a distinct temporal progression pattern across testes developmental days, based on the overall RNA modification landscapes. Notably, the tRNA modification signature exhibited the most robust performance in distinguishing different developmental stages (Figure 1E). In addition, the cluster performance was slightly enhanced by using the PCA contribution top four modifications (m¹G, m²G, m⁵Um, hm⁵C for total RNA; Ψ, Am, Cm, ac⁴C for small RNA fractions) (Figure S1C and

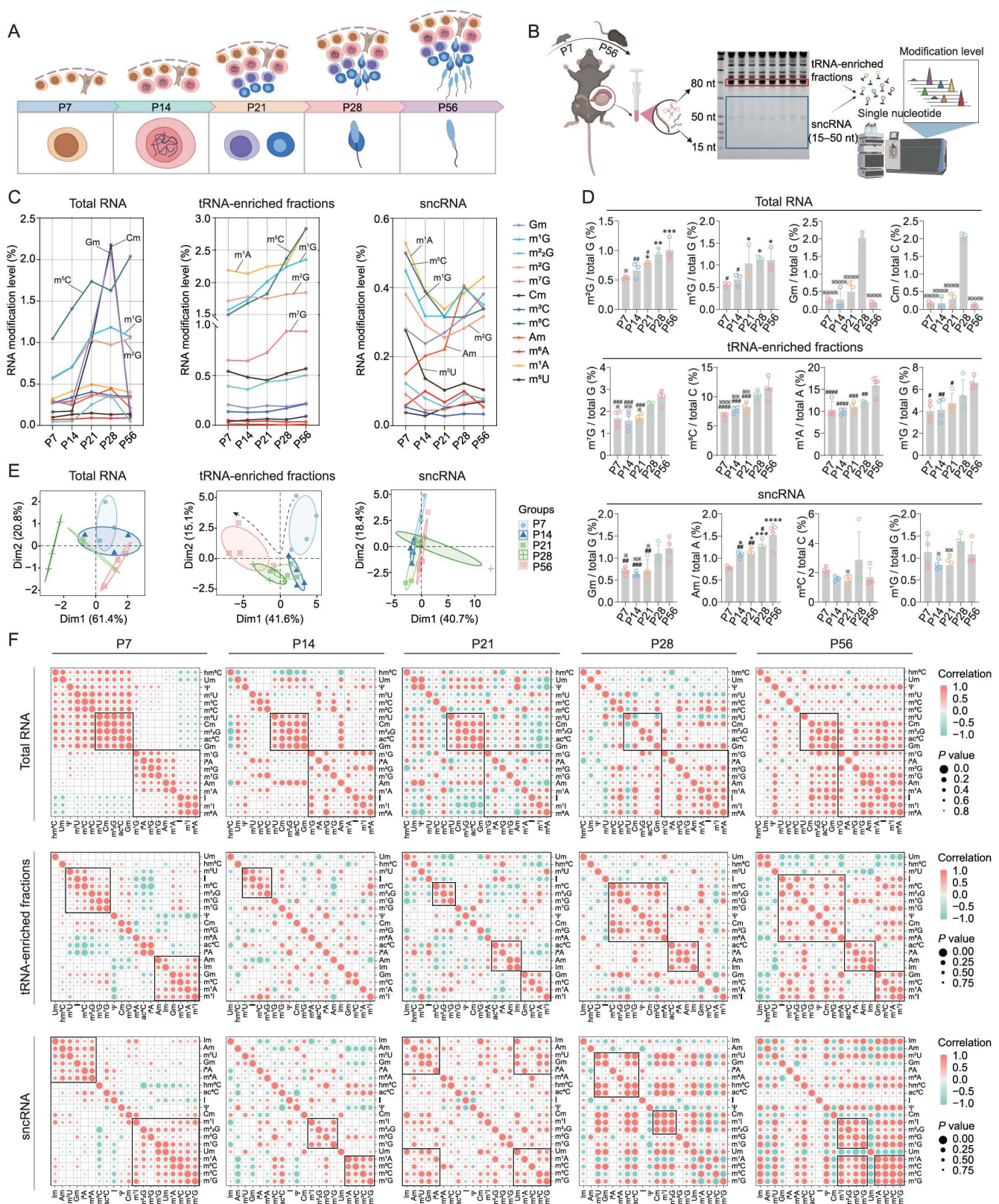


Figure 1 RNA modification profiles and correlations during mouse testes maturation. **A**, Cell types in the developing mouse testis at postnatal days (top) and newly emerging cell types (bottom). **B**, Schematic overview of the experimental workflow for identifying RNA modifications. **C**, 12 types of RNA modification level across P7–P56 mouse testes (the specifically marked RNA modifications denote the most strongly changed ones). **D**, Modification rate of m²G, m¹G, Gm, and Cm in total RNA ($n=3$), modification rate of m⁷G, m⁵C, m¹A, m¹G in tRNA-enriched fractions ($n=4$) and modification rate of Gm, Am, m⁵C, and m¹G in small RNA fractions ($n=4$). Significance: “***” vs. P7, “*” vs. P28, “#” vs. P56; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$. **E**, PCA of P7, P14, P21, P28, P56 mouse testes RNA modification abundance. **F**, Correlation matrices displaying pairwise correlations among RNA modifications for total RNA, tRNA-enriched fractions, and small RNA fractions across P7–P56 mouse testes.

E). These observations indicate a developmental stage-specific RNA modification landscape in testes, which might result from the sequentially emerging spermatogenic cells. Additionally, we previously reported that some modifications, like m¹G, m⁵C, and m²G, exhibited significant co-expression patterns in human sperm and were closely related to sperm motility (Guo et al., 2022), indicating that inter-modification cross-talk might exist during spermatogenesis. Therefore, we performed correlation analyses between different modifications throughout the testis developmental stages. As expected, we identified various RNA modification correlation patterns across total RNA, tRNA-enriched and small RNA fractions in testis (Figure 1F). Interestingly, each developmental stage exhibits distinct inter-modification cross-talk patterns. These correlation features follow a reprogramming trend that gradually disrupted and then re-established along with postnatal days (Figure 1F), which are conserved among total RNA, tRNA-enriched and small RNA fractions. Moreover, correlations between RNA modifications—such as Gm, ac⁴C, m²G, Cm, and m³U in total RNA; Gm, m³C, m¹A, and m¹I in tRNA-enriched fractions; and m¹A, m⁵C, m³C, and m⁷G in small RNA fractions—exhibit a consistent positive relationship along with testicular development, while correlations among others underwent dramatic reconfiguration during this process (Figure 1F). Specifically, the relationship between m⁵U-m⁵C shifts from P7 positive to P56 negative in total RNAs, while m²G and I show positive correlation before P14, and lose correlation at P21, then re-establish stable correlation from P28 to P56 (Figure 1F). Notably, the P21 testes show the most distinct inter-modification cross-talk pattern compared to other stages in total RNA, while in tRNA-enriched fractions, the co-expression patterns are extensively reshaped during testicular development.

Testicular spermatogenic cells exhibit cell-type specific diversity across various RNA modifications.

To further investigate the dynamics of RNA modifications across different spermatogenic cells (Figure 2A), we isolated spermatocytes at pachytene (MPI) and metaphase II (MII) stages, round spermatids (RS) from adult male mouse testes using fluorescence-activated cell sorting (FACS) (Figure 2B). The identity and extent of homogeneity of each sorted cell population were assessed after Hoechst staining and visualized by fluorescence microscopy (Figure 2C). Testicular Sertoli cells and mature sperm from the cauda were also collected. Similarly, we found that tRNA-enriched fractions harbored the highest modification ratio across different spermatogenic cells in testes (Figure 2D; Figure S1A). Total RNA and small RNA fractions exhibited comparable modification ratios on A base, while small RNA fractions showed overall higher modification ratios across U/C/G bases compared to total RNA. Across spermatogenic development, A/C/G base modifications rise progressively through meiotic prophase I (MPI) and meiotic prophase II (MII) stages to peak in RS cells, whereas mature spermatozoa show drastic depletion of these modifications. Conversely, Sertoli cells demonstrate predominant accumulation of U-class modifications (Figure 2D). Next, PCA analyses demonstrated that the abundance of various RNA modifications across total RNA, tRNA-enriched fractions and small RNA fractions is capable of clustering the distinct germ cell types, with tRNA modification signature exhibiting the best performance (Figure S2A). Additionally, when PCA was per-

formed using the top four modifications that were compared based on their contribution to PCA variance, the cluster performance was significantly improved to distinguish each cell type successfully (Figure 2E). Notably, RNA modification signatures on all three RNA groups exhibit a consistent clustering pattern: MPI and RS cells are grouped more closely, while Sertoli cells show much more similarity to mature sperm (Figure 2E; Figure S2A). Stacked bar charts also showed a similar clustering pattern of RNA modification signature in each cell type among total RNA, tRNA-enriched and small RNA fractions (Figure S2B). We next performed heatmap analyses to compare the relative abundance of individual modifications. The data revealed the dynamic variation signature of RNA modification abundance in each RNA fraction among different cell types (Figure 2F). In total RNA, the abundance of distinct modification clusters peaked at specific cell types: 2-O-methylations (e.g., Am, Cm, Um, Im) reached maximal levels in metaphase II spermatocytes (MII), whereas m³C/m⁵C/m²G modifications peak at RS, and I/m¹A/ac⁴C/m⁵U/m⁷G modifications show the highest abundance in Sertoli cells. Importantly, the majority of RNA modifications abundance in tRNA-enriched fractions gradually increases across Sertoli cells, MPI, MII spermatocytes, peaks at RS, then sharply decreases in mature sperm. Moreover, the mature sperm show the lowest RNA modification abundance across total RNA, tRNA-enriched and small RNA fractions (Figure 2F), indicating a specific modification landscape is shaped in each spermatogenic cell (Figure 2F; Figure S2B and C). Collectively, from the above results, we uncovered the dynamics of cell-type-specific RNA modification signatures within the testicular environment.

Sperm acquired maturation specific RNA modification signature in the epididymis.

Since the overall RNA modification abundance of mature sperm experience substantial loss during the transition from testicular round spermatids to cauda mature spermatozoa (Figure 2F), meanwhile, evidences have shown that sperm could incorporate considerable small RNAs that carrying environmental cues via epididymosomes during epididymal maturation, which lead to remarkable alteration in mature sperm RNA pools compared to testicular sperm (Tomar et al., 2024; Trigg et al., 2021). To determine whether sperm RNA modification landscape undergoes further remodeling during epididymal maturation, we isolated epididymal sperm from caput, corpus, cauda, and vas deferens to evaluate the alteration signature of sperm RNA modification profiles across total RNA, tRNA-enriched and small RNA fractions during epididymal maturation (Figure 3A). Sperm purity was assessed by microscopic examination (Figure S3A) and validated through RT-PCR using specific markers for the spermatozoa (Prm2), the germ line (C-kit) and the somatic cell (E-cadherin) (Figure S3B). Globally, we found that sperm indeed experience extensively remodeling of RNA modification signatures across different maturation stages. Specifically, for sperm total RNA, the majority types of RNA modifications (e.g., m⁶A, m⁵C, m¹A) exhibit gradual removal along with sperm maturation, while for tRNA-enriched fractions, the alteration signature clustered into two converse patterns (Figure 3B and C). One cluster (e.g., Gm, Am, m⁷G) shows similarly low abundance in caput and corpus sperm, then increases remarkably in cauda and vas deferens sperm. On the contrary, the other cluster (e.g., m⁵U,

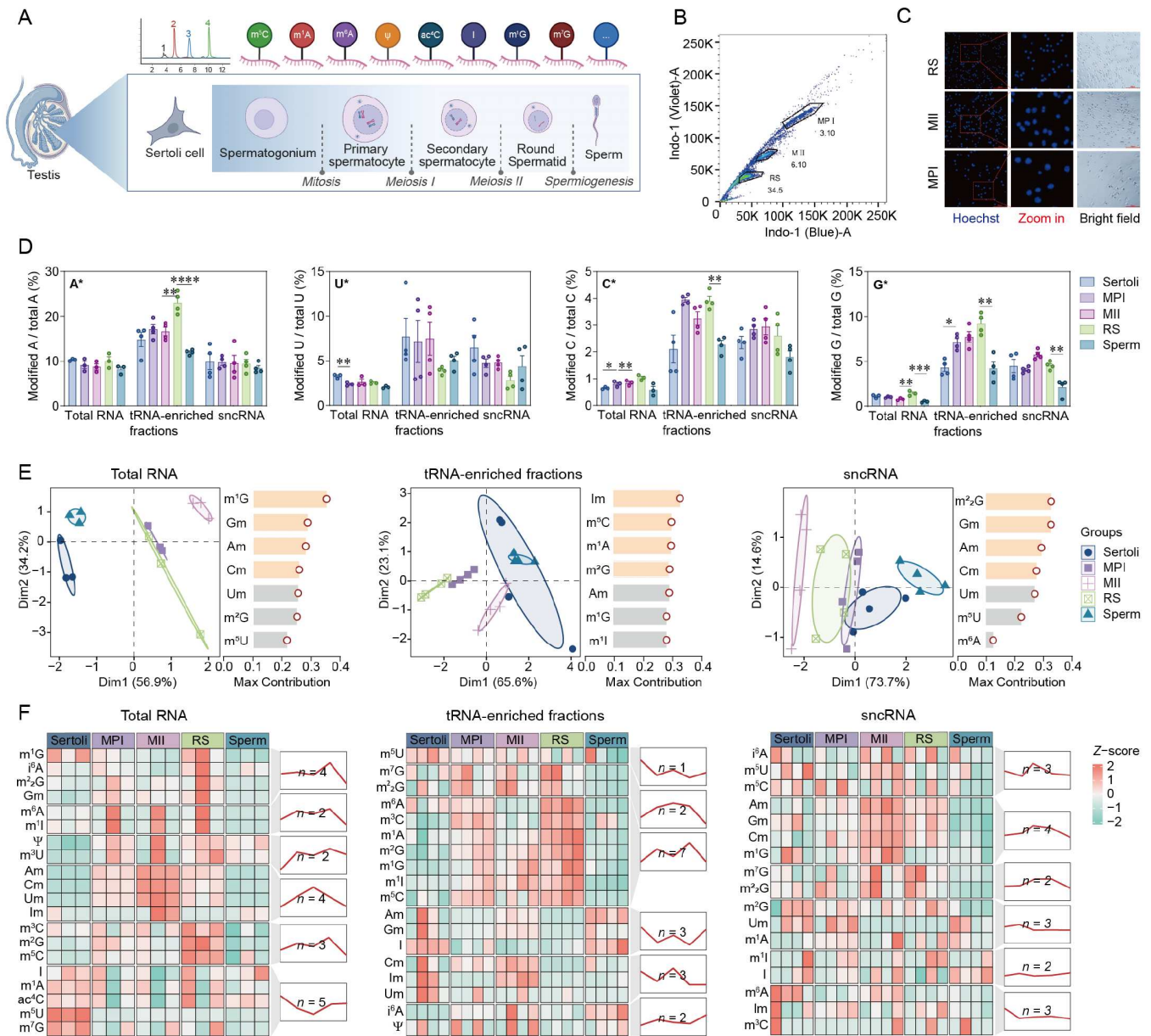


Figure 2 Changes of RNA modifications in mouse Sertoli cells and different spermatogenic cells. **A**, Schematic diagram of modification detection in different types of cells in adult mouse testis. **B**, Isolation of different spermatogenic cells by flow cytometry with signals detected in both Indo Blue and Indo Violet channels. **C**, The MPI, MII and RS cells were examined by Hoechst staining, with an enlarged picture in the middle and a bright field picture on the right. **D**, Proportion of A*, U*, C*, G* modifications in Sertoli cells, different spermatogenic cells and mature sperm on total RNA ($n=3$), tRNA-enriched fractions ($n=4$) and small RNA fractions ($n=4$). A*=sum of all kinds of modifications on base A, U*=sum of all kinds of modifications on base U, C*=sum of all kinds of modifications on base C, G*=sum of all kinds of modifications on base G. Tukey's multiple comparisons test was conducted between adjacent samples. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$. **E**, PCA analysis of different cells (Sertoli, MPI, MII, RS, Sperm) modification presented by the top four modifications with max contribution on total RNA ($n=3$), tRNA-enriched fractions ($n=4$) and small RNA fractions ($n=4$), light orange represents the top four. **F**, Heatmap with trendline of different modifications on total RNA ($n=3$), tRNA-enriched fractions ($n=4$) and small RNA fractions ($n=4$) in Sertoli cells, different spermatogenic cells and mature sperm. Each of the five points on the trend line represents the mean of four replicates at the corresponding time point. In the above results, different cell types are distinguished according to different colors.

m⁵C, m¹A) exhibits the highest level in caput sperm, followed by a gradual decrease along the epididymal segments (Figure 3C). Interestingly, RNA modifications in sperm small RNA fractions show more dynamic regulation, which are clustered into three classes with distinct alteration patterns (Figure 3D). These results reveal the existence of region-specific regulatory mechanisms governing RNA modifications during epididymal maturation. Based on the observed dynamic changes (with caput sperm as the

reference baseline) across total RNA, tRNA-enriched, and small RNA fractions during epididymal maturation (Figure 3B–D; Figure S3C), we further categorized RNA modifications into two groups: RNA modifications that co-decreased in all three RNA groups were termed “down-modifications”, such as m⁵C, m²G, m¹A, and m²G (Figure 3E), whereas those co-increased were termed “up-modifications” (Figure 3F), with m⁵Um as the only up-modification, implying the potential regulatory roles of their RNA modifiers

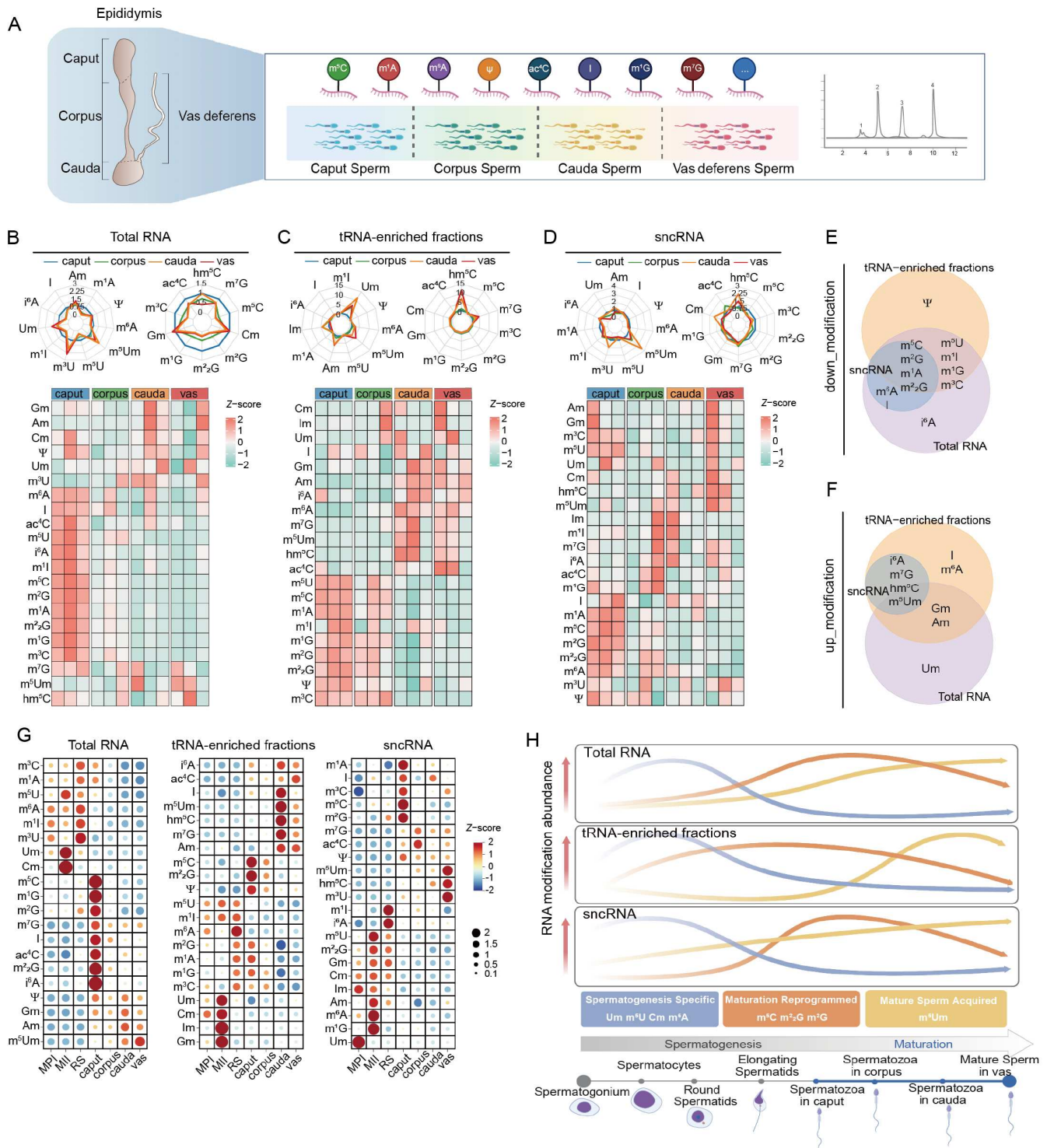


Figure 3 The RNA modification landscape during sperm maturation. A, Quantification of RNA modification abundance in different regions of the epididymis and vas deferens. B–D, Dynamic changes in RNA modification of total RNA ($n=3$) (B), tRNA-enriched fractions ($n=3$) (C), and small RNA fractions ($n=3$) (D) across distinct epididymal segments. E and F, Representative RNA modifications that exhibit decreased (E) or increased (F) expression levels following sperm maturation. G, Global dynamics of RNA modification levels throughout the entire process of spermatogenic cell development. H, Stage-specific fluctuation patterns of RNA modifications during sperm development.

to reshape the epigenetic signature of sperm during epididymal maturation. Additionally, we also observed various inter-modification cross-talk correlations throughout the sperm maturation process, which were more prevalent in total RNA, compared to tRNA-enriched or small RNA fractions (Figure S3D–F).

Divergent RNA modification alteration patterns between spermatogenesis and sperm maturation

To further investigate RNA modification dynamics coupled with spermatogenesis in the testis and sperm maturation in the

epididymis, we performed an integrative analysis spanning from spermatocytes to fully mature spermatozoa and uncovered the distinct stage-specific alterations in RNA modification abundance during this developmental continuum. Overall, C-base RNA modifications show the lowest modification ratio compared to A-, U- and G-base modifications throughout spermatogenesis and sperm maturation. Specifically, as for total RNA, A-base modifications show significant enrichment in testicular spermatocytes, while U-base modifications are preferentially enriched in epididymal sperm. (Figure S3G). A similar trend is also observed in tRNA-enriched fractions (Figure S3G). Additionally, we observed a sharp surge in overall RNA modification levels upon entry into the epididymal caput, followed by divergent trajectories thereafter (Figure S3G). As to each type of RNA modification, we found that RNA modifications with high abundance in testicular spermatocytes display reduction in epididymal sperm, while RNA modifications with low abundance exhibit upregulation in sperm across total RNA, tRNA-enriched and small RNA fractions (Figure 3G), suggesting a divergent RNA modification regulation network during spermatogenesis and sperm maturation. Based on these temporal and spatial expression patterns, we further clustered RNA modifications into three functional categories (Figure 3H). The first group—“spermatogenesis specific modification”—includes modifications (Um, m⁵U, Cm, and m⁶A) that are highly expressed during spermatogenesis but progressively decline after epididymal entry. Notably, m⁶A has been previously shown to promote spermatogonial differentiation (Lin et al., 2017; Xu et al., 2017). The second group—“maturation reprogrammed RNA modification”—comprises modifications (m⁵C, m²₂G, and m²G) that rise sharply upon epididymal entry, peak in the caput sperm, and then gradually decrease during further maturation. The third group—“mature-sperm-acquired modification”—is exemplified by m⁵Um, whose abundance continuously accumulated throughout spermatogenesis and sperm maturation across the epididymis, and peaked at high abundance in vas-deferens sperm (Figure 3G and H). Taken together, our findings demonstrate that the RNA modification landscape in male germ cells undergoes extensive and highly orchestrated reprogramming during both spermatogenesis and epididymal maturation, reflecting stage-specific regulatory demands.

T2DM-induced metabolic stress reprograms sperm RNA modification atlas during maturation.

We previously found that high-fat diet (HFD) exposure could reshape the expression pattern of sperm small RNAs and their associated modifications, which further transmit paternal metabolic disorder to the offspring (Zhang et al., 2018). To further verify whether sperm RNA modification could be reprogrammed upon metabolic stress during sperm maturation in the epididymis, we then established a T2DM mouse model to further characterize the alteration of sperm RNA modification signature during epididymal maturation in response to metabolic stress. The T2DM mice exhibit significantly higher weight gain versus the normal control group (NC) (Figure 4A and B; Figure S4A) with classic T2DM phenotypes: elevated fasting blood glucose, impaired glucose tolerance (GTT), and improved insulin resistance (Figure 4C and D). Although the sperm motility analyses revealed a slight but non-significant trend towards reduced motility in T2DM mice (Figure S4B), PCA of sperm RNA

modification profiles remarkably distinguished T2DM mice from the NC group across sperm total RNA, tRNA-enriched and small RNA fractions (Figure 4E). Interestingly, within the NC group, cauda and vas deferens sperm exhibit a much more similar modification signature compared to caput and corpus sperm at the total RNA level, while in the T2DM group, sperm from caput and corpus show more similarity than sperm from cauda and vas deferens. Moreover, by comparing RNA modification abundance across different epididymal regions (caput, corpus, cauda, vas deferens) between the T2DM and NC group, we identified remarkable alteration of RNA modification profiles across sperm total RNA, tRNA-enriched and small RNA fractions (Figure S4C). Specifically, analysis of sperm total RNA revealed that T2DM induced predominant downregulation of multiple RNA modifications (e.g., Gm, Cm) in the caput epididymis, while causing significant upregulation in the corpus and cauda segment (e.g., m³C, m²G) (Figure 4F; Figure S4C). However, some modifications in tRNA-enriched and small RNA fractions showed consistent downregulation (e.g., m¹I, m¹G, m¹A) or upregulation (e.g., Am, Cm) across sperm in different epididymal segments upon T2DM exposure (Figure 4G; Figure S4C). Additionally, the alterations of RNA modification levels of total RNA and small RNA fractions are more prevalent and significant in caput sperm upon T2DM exposure, while in tRNA-enriched fractions, these alterations are much more significant in vas deferens sperm. Furthermore, the co-expression analyses between different RNA modifications among all epididymal sperm revealed that T2DM significantly reshaped the correlation networks across sperm total RNA, tRNA-enriched and small RNA fractions (Figure S4D–F). For instance, T2DM disrupted the NC-specific positive correlations between m⁶A and m¹A, as well as between m⁶A and m²G, in total RNA modification, while new correlations emerged (a positive correlation between Um and ac⁴C, and a negative correlation between m⁵C and Cm) (Figure 4I). Notably, some correlations remained stable between the two groups, such as the positive association between m¹G and m²G within tRNA-enriched fractions (Figure 4J). In small RNA fractions, the relationship between m⁷G and Cm in small RNA fractions exhibited a polarity shift, changing from positive in NC to negative correlation in T2DM (Figure 4K). These results collectively demonstrate that sperm RNA modifications exhibit maturation-specific alteration dynamics upon T2DM exposure across sperm total RNA, tRNA-enriched and small RNA fractions, which further lead to extensive remodeling of interdependent co-expression networks, indicating a complex adaptive or maladaptive reprogramming of the sperm RNA epitranscriptome under metabolic stress.

DISCUSSION

Beyond the currently well-characterized modifications essential for male fertility—such as m⁶A (Lin et al., 2017; Xu et al., 2017), ac⁴C (Chen et al., 2022b; Zhu et al., 2025) and m⁵C (Blanco et al., 2011)—the regulatory roles of many other specific modifications during spermatogenesis and maturation remain largely unknown. Our study deciphered the overall RNA modifications landscape during testicular spermatogenesis and epididymal sperm maturation across total RNA, tRNA-enriched fractions and small RNA fractions, and uncovered the developmental stages and germ cell types specific RNA modification dynamics. In immature testes (P7), modifications on total RNA primarily

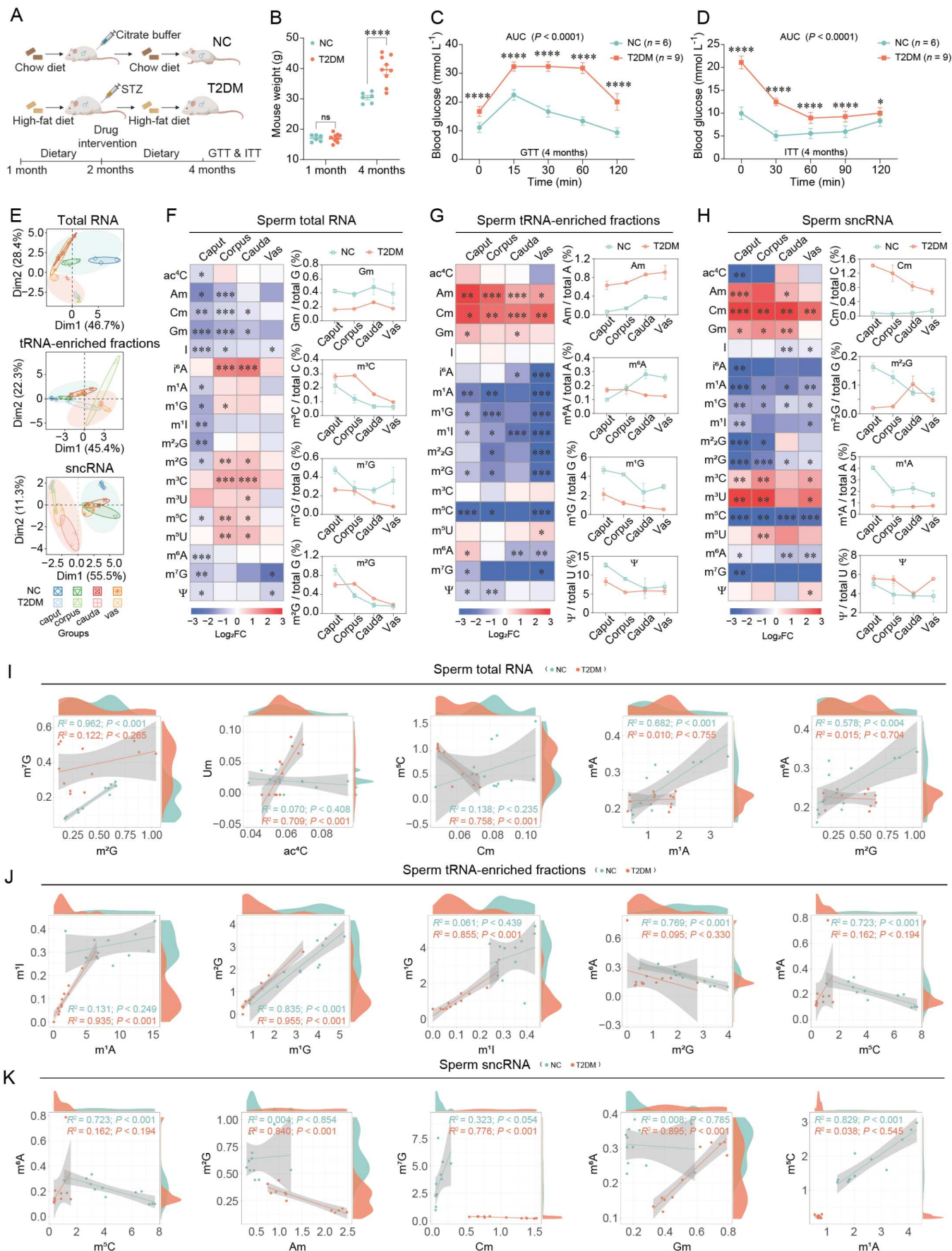


Figure 4 Changes of RNA modifications profile during sperm maturation in T2DM mouse model. A, Schematic diagram of T2DM mouse model construction. B, Comparison of body weight of mice aged one month and four months between NC group ($n=6$) and T2DM group ($n=9$). C, Comparison of GTT blood glucose values and area under the curve (AUC) statistics between NC group and T2DM group during the whole experiment. D, Comparison of ITT blood glucose values and AUC statistics between NC group ($n=6$) and T2DM group ($n=9$) during the whole experiment. n , number of mice in each group, statistical analysis was performed by two-tailed, two-way ANOVA, uncorrected Fisher's LSD. ns, non-significant differences; *, $P<0.05$; **, $P<0.01$; ***, $P\leq 0.001$; ****, $P<0.0001$. Results were shown as the mean standard error of mean (SEM). E, PCA analysis of total RNA ($n=3$), tRNA-enriched fractions ($n=3$) and small RNA fractions ($n=3$) in sperm from different regions of epididymis (caput, corpus, cauda and vas) in NC group and T2DM group ($n=3$). F–H, Differential analysis heatmap of total RNA ($n=3$) (F), tRNA-enriched fractions ($n=3$) (G) and small RNA fractions ($n=3$) (H) in sperm from different regions of epididymis in NC group and T2DM group ($n=3$), statistical analysis was performed by multiple unpaired t tests; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$. The color depth represents $\log_2FC$. I–K, the changes of correlation between different modifications of total RNA (I), tRNA-enriched fractions (J) and small RNA fractions (K) in NC group and T2DM group, and the density maps on the upper and right are the mapping of coordinate values of each point in this direction ($n=12$), the magnitude of correlation is indicated by R^2 , and the significance of change is indicated by P value. In the above results, light orange represents T2DM group and light green represents NC group.

involved bases C and U, whereas in mature testes (P56), modifications shifted to being predominantly on A and G (Figure 1F). In addition, we observed a global pattern disruption and reconstruction of RNA modification cross-talk networks during testis development. The cross-talk between certain modifications exhibits dramatic changes across developmental stages. Since postnatal testes development involves a process of sequentially accumulating spermatogenic cells at various stages (Leblond and Clermont, 1952), these global changes in RNA modification dynamics may result from: (i) stage-specific alterations in testicular RNA expression profiles during germ cell development, and/or (ii) differential expression of RNA-modifying enzymes across developmental stages. Furthermore, these changes could be mediated through: (i) functional interactions between distinct RNA-modifying enzymes, and (ii) spatiotemporal redistribution of RNA modifications on specific RNA substrates.

Notably, the dynamical regulation of certain RNA modifications during spermatogenesis might indicate their indispensable roles during germ cell development, potentially contributing to spermatogonia stem cell maintenance or differentiation to the meiosis process. As reported, m^5C RNA methyltransferase knockout mice exhibit marked reduction of m^5C modification levels in testicular tissue, and exhibit severe spermatogenic defects (such as meiotic abnormalities and spermatid morphological abnormalities) (Guseva et al., 2025; Hussain et al., 2013; Wang et al., 2024). Similarly, we found that the level of m^5C doubled in increase from P7 to P56 (Figure 1C), highlighting its crucial role in spermatogenesis. Like m^5C , we also found that the most RNA modifications on testicular tRNA-enriched fractions were increased continuously from P7 to P56 and formed a clear developmental trajectory upon PCA analysis. This clear temporal trajectory signature of RNA modification abundances in tRNA-enriched fraction probably reflects a stage-specific remodeling of the testicular tRNA repertoire (Figure 1E), highlighting their crucial roles in spermatogenesis. Notably, the composition of total RNA, tRNA-enriched RNA fractions, and small RNAs varies in different germ cells during testis development, which may affect the overall RNA modification signature in testes.

After the spermatogenesis and spermiogenesis in the testis, the spermatid then transits into the epididymis for further maturation. Our data showed that sperm exhibited distinct RNA modification signatures from caput to vas deferens, indicating a unique epigenetic landscape was obtained during maturation along different epididymis regions. Interestingly, the alteration patterns of sperm RNA modification in the epididymis were reprogrammed in T2DM mice. Such alterations might stem from the non-uniform and spatiotemporally specific effects of T2DM stimulus on sperm in the caput, and gradually diminish the differences of RNA modification signature between cauda and

vas deferens sperm. However, T2DM exposure regulates sperm RNA modification signature during epididymis maturation in divergent alteration patterns. For example, some cross-talk between RNA modifications with stable positive correlations (e.g., m^7G - m^2G and m^6A - m^1A on total RNA) was disrupted in the T2DM group (Figure 4I), while the co-expression pattern of m^1G - m^2G on tRNA seems resistant to such metabolic change (Figure 4J). Since T2DM can induce cellular methyl donor S-adenosylmethionine (SAM) deficiency (Walker, 2017), the levels of RNA methylation dependent on SAM supply (such as m^7G) are more susceptible to metabolic imbalance, which might lead to the competitive behavior of modification enzymes for limited substrate, resulting in an overall decrease of RNA methylation levels in sperm. Moreover, such metabolic alteration, potentially mediated by imbalanced methyl donor substrate availability, may further contribute to the observed shift in modification cross-talk patterns—specifically the transition from an uncorrelated to a negatively correlated relationship between m^5C and Cm in total RNA following T2DM exposure (Figure 4I).

Studies have shown intimate cross-talk between sperm and epididymal epithelial cells via epididymal exosomes, which could influence the expression profile of small non-coding RNAs (sncRNAs) during sperm maturation in the epididymis (Sharma, 2019; Trigg and Conine, 2024; Trigg et al., 2021; Wang et al., 2023a). Here, we found that the sperm small RNA modification landscape was also dynamically regulated during sperm maturation in the epididymis. The presence of these RNA modifications and their abundance variation signature might remarkably expand the epigenetic information storage capacity of sperm RNA repertoire under diverse environmental exposures (Naveed et al., 2025; Rando, 2016). Moreover, as the key information of “sperm RNA code” (Chen et al., 2016b), RNA modifications experience large-scale reprogramming during spermatogenesis and sperm maturation. Most RNA modifications exhibit the lowest abundance in mature sperm compared to testicular spermatocyte and caput immature sperm, which might be actively erased or passively diluted during development to minimize the side effect of paternal epigenetic imprints gained from environmental exposure in offspring development. The sharp decline may be linked to cytoplasmic remodeling during spermatid elongation, where the expulsion of modification-rich cytoplasm could contribute to the observed decrease. Additionally, considering the regulatory roles of RNA modifiers, such as Mett13 and Nat10, in spermatogonia proliferation and differentiation during meiosis, their downregulation may help suspend such functions once spermiogenesis is complete, which further leads to the downregulation of RNA modification abundances and biogenesis of small RNA fragments in epididymal sperm. Whether this process is regulated by demethylation activity or

triggered by RNA profile alteration remains largely unknown, and warrants further investigation through integrated analysis of RNA expression and modification data across spermatogenesis and sperm maturation.

While we systematically deciphered the dynamics of overall RNA modification landscapes across different RNA types in testes and epididymal sperm, many new questions are raised based on these discoveries. In addition, due to the low resolution of LC-MS/MS-based RNA detection platform, the current study has its own limitations in several aspects. Firstly, this method was established on the enzymatic digestion of each RNA fraction into mono-nucleotides, thus enabling the detection of various modification types in one sample at the total abundance level, which could not determine the abundance of a specific modification on individual RNA molecules (e.g., tRNA^{Ala}) or specific transcript sites. Secondly, the observed alteration of each RNA modification abundance could not be simply interpreted as the output from the regulation of RNA modification modifiers or the composition of RNAs that possess such RNA modifications. Additionally, our data could not provide the position information of certain RNA modification on certain RNA sequence. Recent advances in high-throughput sequencing have enabled the detection of RNA chemical modifications at single-base resolution. However, most NGS-based approaches are limited to simultaneously detecting multiple types of RNA modification in one RNA sample (Chen et al., 2015; Meyer et al., 2012; Wang et al., 2021; Yang et al., 2024). Consequently, these technologies only enable the viewing of an isolated piece for one certain RNA modification information, failing to grasp the complete picture of the RNA modification cross-talk universe. Nevertheless, based on this LC-MS/MS-dependent RNA quantification platform, we can quantify dozens of RNA modifications simultaneously across total RNA, tRNA-enriched fractions and small RNA fractions during spermatogenesis and sperm maturation. This comprehensive approach has enabled us to systematically characterize the global, dynamic co-variation patterns of the sperm RNA modification landscape. Moreover, the calculated modification ratio in each type of RNA fraction, such as m⁶A/A-base on total RNA, reflected the overall modification tendency that potentially correlated with the abundance and enzyme activity of RNA modification modifiers, indicating an active regulation state of specific modification during spermatogenesis and sperm maturation.

Additionally, nanopore-based sequence technology exhibits promising application on simultaneously profiling multiple RNA modifications with single RNA sample, while due to the intrinsic high complexity of RNA modifications and enormous calculation problems, there is still no available nanopore sequencing strategy that could effectively and precisely profile multiple types of RNA modification at sequence or single-base resolution within one RNA sample across total RNA and small RNAs (Alfonzo et al., 2021; Leger et al., 2021; Ueda et al., 2023). Nevertheless, combined transcriptome sequencing and site-specific RNA modification profiling technologies are still of great promise that should be applied in future investigations, which could provide a more precise epitranscriptomic landscape with various RNA modification information at single-base resolution to elucidate their regulatory roles and molecular mechanisms along spermatogenesis and epididymal maturation. Moreover, precise manipulation of RNA knockdown can be achieved by a CRISPR/RfxCas13d-mediated strategy, allowing functional investigation of certain RNAs (Zhang et al., 2024). This system can be further

combined with RNA writer or eraser enzymes to investigate site-specific RNA modifications, thereby dissecting the functional contribution of the modification itself during spermatogenesis. For example, the ADAR deaminase domain fused to dCas13 enables programmable adenosine-to-inosine (A-to-I) RNA editing in selected transcripts (Wang et al., 2025). Alternatively, dCas13b fused with FTO utilizes FTO's dioxygenase activity to achieve site-specific erasure of m⁶A modification (Kang et al., 2024). Collectively, our study delineates the dynamic features of RNA modifications during spermatogenesis and sperm maturation, demonstrating the trajectory origins of disrupted RNA modification homeostasis in sperm under T2DM stress, and providing an RNA modification resource for investigating the underlying mechanisms of RNA modification-mediated epigenetic reprogramming during testes development and sperm maturation.

MATERIALS AND METHODS

Animals

The C57BL/6 mice were purchased from Vital River Laboratory Animal Technology Company (Beijing, China). The animals were raised under controlled conditions with water and standard laboratory feed, maintained on a 12-h light/12-h dark cycle, with temperature at 22–25°C and relative humidity between 40%–60%.

Type 2 diabetes mellitus mouse model

The T2DM mouse model was established, referring to a previous study (Chen et al., 2022a; Deeds et al., 2011; Heydemann, 2016). Briefly, male mice aged 4 weeks were fed on a high-fat diet (Rodent diet with 60 kcal% fat, Research Diets Inc., USA, D12492) for 1 month, then injected intraperitoneally with streptozotocin (STZ, Sigma-Aldrich, USA, V900890, dissolved in sodium citrate) at a dose of 150 mg/kg. After that, continually fed with a high-fat diet for another 2 months. Mice in the normal control group (NC group) were fed on a normal diet (BEIJING HFK BIOSCIENCE CO., LTD., Beijing, China, Diet 1415) from 4 weeks of age for 3 months, with the injection of the same volume of sodium citrate at 1 month. The body weight of these mice was recorded every month.

GTT and ITT

For GTT (glucose tolerance test) experiments, T2DM mice were fasted for 15 h and NC mice were fasted for 6 h, with food removed but water available *ad libitum*. The baseline blood glucose was measured in duplicate using a glucometer and test strips (ONETOUCH UltraEasy, LifeScan, USA). Mice then received an intraperitoneal injection of a sterile glucose solution at a dose of 2 g/kg body weight. Blood glucose was subsequently measured at 15-, 30-, 60-, and 120-min postinjection.

The mice were subjected to ITT (insulin tolerance test) experiments after receiving the GTT for 1 week. After the baseline blood glucose levels were measured, the mice received an intraperitoneal injection of insulin at a dose of 0.75 IU/kg (Aladdin, Shanghai, China, 12584-58-6). Blood glucose levels were subsequently measured at 30-, 60-, 90-, and 120-min post-injection.

Isolation of meiotic prophase I, II spermatocytes and round spermatids from testes by Flow cytometry

Testicular single-cell suspension of adult mouse was performed as follows. After removal of the tunica albuginea of the testes, the seminiferous tubules were dissociated with forceps and transferred into 5 mL of Dulbecco's Phosphate-Buffered Saline (DPBS, Corning, USA, 21-031-CV). The tubules were then gently triturated by pipetting, followed by centrifugation at $500\times g$ for 5 min. Resuspended the cell pellet in DPBS and washed twice. Added 5 mL of TrypLE™ Express Enzyme (Thermo Fisher Scientific, USA, 12604039) to the pellet and incubate at 37°C for 10 min with intermittent pipette mixing. Digestion was terminated by 5 mL of complete cell culture medium. The cell suspension was filtered through a $70\text{-}\mu\text{m}$ cell strainer (BIOLOGIX, Shandong, China, 15-1070) and centrifuged at $500\times g$ for 5 min. Resuspended the pellet with 5 mL DPBS and placed the cells on ice for further spermatocyte isolation.

MPI, MII, and RS sorting and data acquisition were performed using a BD FACS Aria II flow cytometer (BD Biosciences, USA) according to previous research (Gaysinskaya et al., 2014). Briefly, testes cell suspension was stained with Hoechst33342 (Beyotime Biotechnology, Shanghai, China, C1028) at 37°C for 20 min in a concentration of $6\text{ }\mu\text{g}$ per million cells. Removed the Hoechst dye and resuspended the cells in DPBS before sorting. The flow cytometry flow rate was optimized to 1,000–3,000 events/s. Forward scatter (FSC-A) and side scatter (SSC-A) parameters were analyzed to exclude debris particles from the cell by setting gates. Hoechst fluorescence was excited by a 355 nm UV laser, with emission signals detected in both Indo Blue (670/50 nm) and Indo Violet (450/50 nm) channels. When necessary, a gating strategy based on the Hoechst Blue/Hoechst Red fluorescence plot was applied in conjunction with the FSC/SSC plot. This approach allowed effective discrimination of cells based on both Hoechst dye fluorescence and light scatter parameters. MPI, MII, and RS were sorted into 5 mL round-bottom flow cytometry tubes (Falcon, USA, 08-771-23) containing 1 mL of DPBS.

Isolation of Sertoli cells from testes

The isolation of Sertoli cells from the testis was performed as previously reported (Hemendinger et al., 2002; Scarpino et al., 1998; Teng et al., 2005). Briefly, the testes from adult male mice were cut into small pieces after removing the testicular tunica albuginea. Added 5 mL DPBS to disperse the seminiferous tubule mass, and washed twice with DPBS. The seminiferous tubule mass was sequentially digested by different enzymatic buffer systems: (i) 3 mL digestive buffer I (DMEM: F12 contains 100 U/mL collagenase and 0.05% Dnase I) at 37°C for 20 min; (ii) 3 mL of digestive buffer II (DMEM: F12 contains 100 U/mL collagenase, 0.1% hyaluronidase (Merck, Germany, H3757-100MG) and 0.05% Dnase I) at 37°C for 15 min; (iii) 3 mL digestive buffer III (0.25% trypsin (Gibco, USA, 12604021) containing 100 U/mL collagenase, 0.1% hyaluronidase and 0.05% Dnase I) at 37°C for 15 min. After each digestion buffer system, washed the cell pellets twice with DPBS. The digestion was ended by adding an equal volume of complete cell culture medium. Washed the cells twice with DPBS and sieved with a $70\text{ }\mu\text{m}$ cell filter to remove tissue debris. Collected the cells by centrifugation at $500\times g$ for 5 min. Cultured the cells in a 10 cm dish for 5 d and changed the

medium once every 2 d to remove the unadhered cells. The cells that adhered to the dish wall were collected as Sertoli cells.

Epididymal sperm collection and purification

Given the low sperm yield from segments of the epididymis, sperm from bilateral epididymides of 2–3 adult male mice were pooled as one biological replicate. The epididymis and vas deferens were isolated from the adult male mice, with removal of adipose tissue and blood vessels. The caput, corpus, and cauda regions of the epididymis, along with the vas deferens, were separated from each other and incubated in DPBS at 37°C for 10 min to release the sperm from the caput and cauda epididymis by using a 1 mL syringe needle. For the corpus and vas deferens, sperm were gently extruded from the lumen using fine-tipped forceps. The removal of somatic cell contamination followed our previous publication (Chen et al., 2016a). Briefly, the released sperm from each segment were dispersed into 2 mL of DPBS in a 35 mm culture dish at 37°C for 10 min, then filtered through a $40\text{ }\mu\text{m}$ cell strainer (BIOLOGIX, 15-1040) and loaded into 10 mL of pre-chilled somatic cell lysis buffer (0.1% SDS, 0.5% Triton X-100, 1% DEPC-treated water). The sperm suspension was gently mixed and placed on ice for 40 min to remove somatic cell debris. Due to the highly resistant structure of mature sperm—which includes tightly interwoven plasma membrane, acrosomal membrane, and nuclear envelope—the somatic lysis buffer effectively removed somatic contaminants without compromising sperm integrity. Collected the sperm by centrifugation at $600\times g$ for 5 min at 4°C and resuspended the sperm in 1 mL DPBS. The purified sperm pellet was washed three times with 1 mL of DPBS before RNA extraction. The purity of the sperm samples was determined by microscopic examination.

RNA extraction and size selection for different RNA fractions

Testes from mice at postnatal days 7, 14, 21, 28, and 56 were collected. After removal of the testicular tunica albuginea, the cells were homogenized with TRIzol reagent (Thermo Fisher Scientific, 15596018) for RNA extraction according to the manufacturer's protocol. The isolated testicular cells were directly placed in TRIzol reagent for RNA extraction. For the sperm RNA extraction, the collected sperm pellet was lysed in 200 μL of sperm lysis buffer (2.13 mol/L guanidine hydrochloride, 31.3 mmol/L EDTA, 41.3 mmol/L Tris, 6.66% Tween-20, 1.67% Triton X-100, 66 mmol/L DTT, and 27.2 U/mL proteinase K) as previously reported (Griffin, 2013). The sperm lysate was incubated on ice for 5 min until the solution became clear. Added three times the volume of TRIzol™ LS reagent to the sperm lysis for RNA extraction. The extracted RNAs were dissolved in nuclease-free water (Thermo Fisher Scientific, 10977015).

The isolation of different-sized RNA fractions from total RNA followed our previous study (Shi et al., 2021). Briefly, the RNA mixed with $2\times$ RNA loading dye (NEB, USA, B0363S) was linearized at 95°C for 5 min and immediately placed on ice. Then separated the RNAs by running 15% 7 mol/L Urea-PAGE gel. The gels that contained RNA fractions around 80 and 15–50 nt were excised based on small RNA ladders (NEB, N0364S). 400 μL of RNA gel extraction buffer (300 mmol/L sodium acetate, 0.25% SDS, 1 mmol/L EDTA) was added to the gels and incubated at

65°C with shaking at 1,200 r/min for 10 min. The samples were then snap-frozen in liquid nitrogen and thawed twice. The gel lysis was incubated overnight on a rotator at room temperature and centrifuged to collect the RNA supernatant. Added 3 times absolute ethanol along with 2 µL of glycogen to the collected RNA supernatant for RNA precipitation at −20°C overnight. The RNA was pelleted by centrifugation at 16,000×*g* for 15 min at 4°C and resuspended in nuclease-free water. Stored at −80°C for further experiments.

RT-PCR

1 µg of total sperm RNA was reverse transcribed using 4× gDNA Wiper Mix and 5× HiScript III qRT SuperMix (Vazyme, Nanjing, China, R323-01) according to the manufacturer’s protocol. The cDNAs were used as the template for PCR amplification of epithelial cell marker E-cadherin, spermatogonium cell marker c-KIT, and sperm-specific marker PRM2 by using 2× GoTaq® Master Mix (Promega, USA, M7123) following the previous PCR program. The primers used are displayed in Table 1, and the PCR program is detailed in Table 2. PCR products were loaded onto a 2% agarose gel prepared with agarose (BBI, Shanghai, China, A620014-0100). Images captured by the Multifunctional gel image analysis system (Tanon MINI Space).

Detection of RNA modifications abundance by LC-MS/MS

The LC-MS/MS-dependent RNA modification detection experiments were performed according to our previously reported procedure with slight modifications (Guo et al., 2023). Briefly, A total of 50–100 ng RNAs were firstly digested in a 40-µL reaction system containing 4 µL 10 × Nuclear P1 Buffer, 10 IU Nuclear P1 (NEB, M0660S), 37°C for 1 h. Next, added 1 IU benzonase (Sigma-Aldrich, E8263), 2 IU alkaline phosphatase (Sigma-Aldrich, P5521), 0.04 IU phosphodiesterase I (USB, 20240Y) and 5 µL 10× rCutSmart (NEB, B6004S) to a 50-µL volume at 37°C for 2 h. Then, removed the enzymatic system using a Nanosep 3K device with Omega membrane (Pall Corporation, USA, OD003C34). The LC-MS/MS was performed by Waters ACQUITY- UPLC I-class carrying TRIPLE QUAD 6500+ mass spectrometry system and column model of MS is ACQUITY UPLC HSS T3. The MS system was operated in a positive ion mode using a multiple reaction monitoring (MRM) scan model. The detailed mass transition parameters were monitored in Table S1. The mobile phase consists of 0.1% formic acid in water and acetonitrile, with a flow rate of 0.3 mL/min. The gradient program is as follows: 0 min: 100% 0.1% formic acid in water, 0% acetonitrile; 7.65 min: 99% 0.1% formic acid in water, 1% acetonitrile; 9.35 min: 94% 0.1% formic acid in water, 6% acetonitrile; 11 min: 90% 0.1% formic acid in water, 10% acetonitrile; 12 min: 80% 0.1% formic acid in water, 20% acetonitrile; 14 min: 50% 0.1% formic acid in water, 50% acetonitrile; 17 min: 25% 0.1% formic acid in water, 75% acetonitrile; 17.5–30 min: return to 100% 0.1% formic acid in water, 0% acetonitrile until the next sample.

Mass spectrometry data analysis

The raw data were acquired by Analyst 1.6 software, then processed and analyzed by SCIEX OS software. The molar concentration of each RNA modification was quantitatively

Table 1. PCR primers for sperm/somatic cell markers

Primer	Sequence (5'-3')
E-cadherin_F	GAACCTGGTTCAGATCAAAT
E-cadherin_R	GAATAGAGGATGTACTTGGC
Prm2_F	CAGCCACAAAAATCCACCTG
Prm2_R	GACGGCAGCATCTTCGCC
c-KIT_F	CAACAAAGAGCAAATCCAGGCC
c-KIT_R	CACCAGCTCCCAATGTCTTT

Table 2. RT-PCR program

Temperature	Time	Cycles
94°C	5 min	
94°C	30 s	
58/60°C	30 s	×35
72°C	30 s	
72°C	5 min	

calculated according to the standard curve run for the same batch of samples. The percentage of each modified ribonucleoside was normalized to the total amount of quantified ribonucleosides with the same nucleobase, respectively. For example, the percentage of m⁶A=Molar Concentration (m⁶A)×100%/Molar Concentration (m⁶A+Am+A+m¹A+I+Im+m¹I+i⁶A), the percentage of m⁵C=Molar Concentration (m⁵C)×100%/Molar Concentration (m⁵C+Cm+C+ ac⁴C). As to the relative percentage of modified and unmodified four nucleotides (adenine, uracil, cytosine, and guanine) in total RNA, tRNA-enriched fractions, and 15–50 nt small RNA fractions, the sum of all modified and unmodified nucleotide abundance was set to 100, and the percentage was calculated by the average of each nuclear base.

Data analysis

The PCA uses the “prcomp” function of R Stats Package by adopting the standardized method of log₂ or Z-score. For *t*-test, variance analysis uses unpaired multiple *t*-test and one-way ANOVA multiple comparisons, data were shown as the mean standard error of mean (SEM) in GraphPad Prism 10; The correlation analyses between modifications were applied the “corr.test” function of R (version 4.3.3) corrplot package or Pearson’s correlation test in the “stat_correlation” function of ggpmisc package (T2DM correlation analysis) to calculate the correlation coefficient (*r*). The statistical difference *P*-value<0.05 is considered statistically significant. Detailed Log₂FC and *P*-value for modification analyses for Figure 4F–H were provided in Table S2. Heatmap visualization can be performed using the ComplexHeatmap package or ChiPlot Online, with Z-score normalization and clustering methods including mfuzz or complete-linkage clustering.

Data availability

Data supporting the findings of this study are available within the article and its Supplementary Material. Additional data are available from the corresponding author upon reasonable request. Raw data are provided in this article.

Compliance and ethics

The authors declare that they have no conflict of interest. All animal experimental procedures

conducted in this study were approved by the Tongji University Animal Care and Use Committee (Approval No. TJAB07623101) in accordance with ethical guidelines.

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Supporting information

The supporting information is available online at <https://doi.org/10.1007/s11427-025-3134-1>. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.

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