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DICER1 regulates antibacterial function of epididymis by modulating transcription of β-defensins

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DICER1 is a key enzyme responsible for the maturation of microRNAs. Recent evidences suggested that DICER1 and microRNAs expressed in epididymis were involved in the control of male fertility. However, the exact mechanism remains to be elucidated. Here, we created a mouse line by targeted disruption of *Dicer1* gene in the principal cells of distal caput epididymis. Our data indicated that a set of β-defensin genes were downregulated by DICER1 rather than by microRNAs. Moreover, DICER1 was significantly enriched in the promoter of β-defensin gene and controlled transcription. Besides, the antibacterial ability of the adult epididymis significantly declined upon *Dicer1* deletion both *in vitro* and *in vivo*. And a higher incidence of reproductive defect was observed in middle-aged *Dicer1*$^{-/-}$ males. These results suggest that DICER1 plays an important role in transcription of β-defensin genes, which are associated with the natural antibacterial properties in a microRNA-independent manner, and further impacts the male fertility.

Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNAs (21–24 nucleotides) acts as the regulator of translation initiation and mRNA stability (Bartel, 2018). In mammals, two key enzymes participate in the biogenesis of canonical miRNAs: the RNase III DROSHA executes the nuclear processing of primary miRNAs (pri-miRNAs) with DiGeorge syndrome chromosomal region 8 (DGCR8) to produce pre-miRNAs, the RNase III DICER cleaves the pre-miRNA hairpin into mature miRNAs in the cytoplasm (Bartel, 2018). Then one single-strand of mature miRNA is incorporated into the miRNA-induced silencing complexes (miRISCs), interacts with mRNA targets that have similar sequences and silences the gene expression (Jonas and Izaurralde, 2015). Quantitative studies have suggested that the miRNAs establish an expression threshold for their target genes to provide a safety mechanism against random fluctua-

tions and transient signals (Legewie et al., 2008; Levine and Hwa, 2008). Therefore, dysregulation of miRNAs induces the disorder of functional gene expression, which leads to dysontogenesis and pathogenesis. A great number of indications reveal that miRNAs are involved not only in the development of different organs including hearts, skeletal muscles, brains, kidneys, lungs, livers and pancreas but also in various diseases such as cardiac hypertrophy, rhabdomyosarcoma (Sayed and Abdellatif, 2011). Recently, the miRNA is also involved in male reproductive system. miRNAs in mouse testis were reported to differentially express from pre-puberty to adulthood (Yu et al., 2005). Deletion of testicular miRNAs, such as the *miR-34b/c* cluster and the *miR-449* cluster, was noted to impair spermatogenesis and male fertility (Comazzetto et al., 2014; Wu et al., 2014). However, the source, function and molecular mechanism of miRNAs in the reproductive system are largely unknown.

The epididymis is a male reproductive organ. In mammals, spermatozoa are produced in the testis. However, it is often overlooked that testicular sperm cannot swim to recognize and fertilize ova until they undergo a programmed maturation process during their migration through the epididymis. The epididymis can be roughly divided into the caput, corpus and cauda

regions (Cosentino and Cockett, 1986). More precisely, for example, the epididymis of mouse can be divided into 10 intraregional segments (Jelinsky et al., 2007). The principal cell is the main cell type found in the epididymis of all mammals. Within these columnar epithelial cells, protein expression and secretion is segment-dependent and tightly regulated, therefore characterizing a special luminal fluid to protect and modify sperms (Johnston et al., 2005; Jelinsky et al., 2007). Actually, 70%–80% of proteins secreted into the epididymal lumen are from the caput region (Cornwall, 2009). The gene expression in principal cells of caput epididymis is essential for sperm maturation. However, the molecular mechanisms involved in the control of regionalized gene expression are not fully understood.

It is well established that the gene expression in the epididymis is regulated by several factors, including hormones, transcription factors and paracrine factors in a region-specific manner (Belleannee et al., 2012b). Recent evidence has revealed a novel and extremely important role of non-coding RNAs and DICER1 in the regulation of epididymal gene expression. Our studies have documented the presence of miRNAs in human epididymis and several are differentially expressed in newborn and adult (Zhang et al., 2010). The comparative profiling of epididymal miRNAs has revealed the distinct temporospatial patterns in human and mouse epididymis (Zhang et al., 2010; Belleannee et al., 2012a). Therefore, by regulating target genes, miRNAs may play roles in the proliferation of epitheliums, the development and maintenance of epididymis (Ma et al., 2012). Moreover, the deficiency of DROSHA or DICER1 usually leads to the depletion of bulk of miRNAs (Kim et al., 2016). Some studies have shown that the conditional knockout of *Dicer1* in the proximal caput epididymis results in development failure associated with dedifferentiated epithelium and instability of the sperm membrane and reduced ability of fertilize the oocyte (Bjorkgren et al., 2012, 2015). Related gene expression changes are triggered by miRNAs in this distinct region (Jerczynski et al., 2016). Besides, *Dicer1* is not restricted to the miRNA processing. DICER1 is involved in removing the accumulated endogenous dsRNA and contributes to the integrity of rDNA (Sinkkonen et al., 2010; White et al., 2014). The deficiency of *Dicer1* haltes the glowing of embryonic stem cells (ES cells) (Kanellopoulou et al., 2005); however, *DGCR8*-null ES cells do not stop glowing and differentiation although DGCR8 is essential for miRNA biogenesis (Wang et al., 2007). The phenotypic difference suggests that DICER1 regulates the differentiation of ES cells without miRNAs. Similar incidents occur in other tissues. DICER1 is required by murine follicular development, but DROSHA is dispensable (Yuan et al., 2014). By comparing the non-overlapping functions between the main components in miRNA processing approaches, the role of DICER1 in retrotransposon transcript degradation is revealed that losing of DICER1 induces Alu RNA toxicity which is related with macular degeneration (Kaneko et al., 2011). The particularly intriguing point is the genome-wide interaction of DICER1 with RNA polymerase II (POL II) (White et al., 2014). It may suggest the function of DICER1 in regulating gene transcription. A research work has shown that double hydrogen testosterone (DHT) can stimulate

DICER1 to occupy the promoter of prostate-specific antigen gene (Redfern et al., 2013). But the detailed mechanism is unknown. The miRNA-independent fuctions of DICER1 and DROSHA in epididymis are still to be explored.

In this study, we have created a conditional gene knockout mouse line, in which *Dicer1* was deleted in the principal cells of distal caput epididymis (epididymal segment IV & V, CAP4/5) by using *Lcn5-Cre-loxP* system. We assessed the role of DICER1 in male fertility and antibacterial ability of epididymis. The *Dicer1*-null mice showed a phenotype resembling the human disease epididymitis and concomitant infertility. Furthermore, we used multiple genome-wide sequencing data to obtain the maps of miRNAs and mRNAs in the epididymis of adult *Dicer1*$^{-/-}$mice. By analyzing these expression profiles and functional studies, we revealed a set of β-defensin genes transcriptional controlled by DICER1 with the miRNA-independent manner in epididymis. This is the first demonstration that DICER1 regulates the antibacterial ability of epididymis through controlling the transcription of β-defensin genes in epididymial cells directly and influences the latter male fertility. This study likely contributes to understand the significance of DICER1 expressed in principal cells in maintaining the epididymis homeostasis and causes of epididymitis.

Results

Dicer1 is highly expressed in the caput region of adult mouse epididymis

Dicer1 is known to express persistently in the epididymis of mouse from birth into postnatal day 42 (Bjorkgren et al., 2012), but its tissue- or region-specific expression pattern in adult mouse is ambiguous. We profiled the *Dicer1* mRNA expression of multiple organs by real-time quantitative PCR (qPCR) and found the highest level of *Dicer1* mRNA in the caput region of epididymis (caput EP) in 2-month-old (2 M) mouse (Figure 1A). Immunohistochemical staining also showed a protein expression trend in accordance with mRNA (Figure 1B). Moreover, DICER1 protein was wildly located in epididymal epithelium (Figure 1C).

Dicer1 is conditionally disrupted in the CAP4/5 of mouse epididymis

Since the systematic knockout of *Dicer1* is embryonic lethal, we generated a conditional knockout mouse line in which *Dicer1* was ablated in the CAP4/5 of adult epididymis by crossing the *Dicer1*$^{fl/fl}$ mice with the *Lcn5-Cre* mice (Xie et al., 2013). To verify the spatial and temporal disruption of *Dicer1* gene, genomic DNA from the epididymis and several other organs were analyzed by PCR using the specific primer pair DicerF1 and DicerDel1 which bind to the sequence near flox sites (Figure 2A). The recombination of *Dicer1* was observed in 30-day-old *Dicer1*$^{-/-}$ mice and reached maximum in 60-day-old *Dicer1*$^{-/-}$ mice (Figure 2B). The deletion of *Dicer1* occurred specifically in CAP4/5 (Figure 2C). The *mT/mG* mouse line can be used as a tool for visualization of CRE activation. We crossed *mT/mG* mice with *Dicer1*$^{-/-}$ mice. The triple transgenic progeny demonstrated a powerful CRE activation in CAP4/5 (Figure 2D) and a dramatically upregulated *Cre* mRNA level (Figure 2E). And
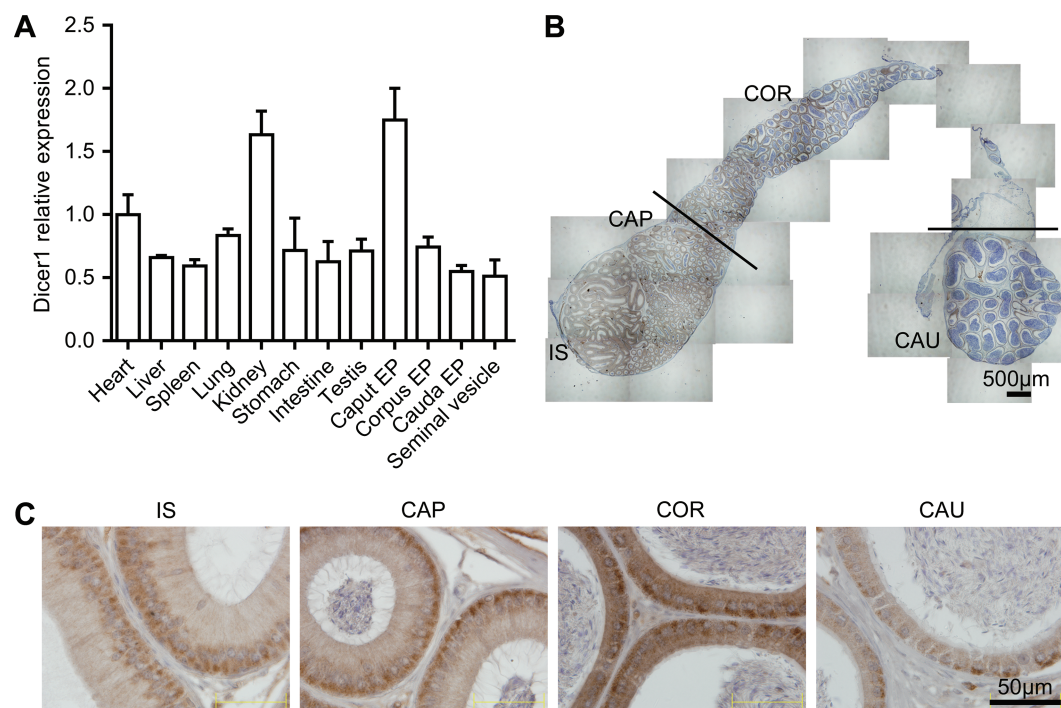
Figure 1 DICER1 expressed highly in the caput of epididymis. (**A**) RT-qPCR analysis of *Dicer1* mRNA in different organs of the mouse. The expression of *Dicer1* in heart is set as 1. Data in the bar graph are expressed as mean ± SEM (*n* = 3). (**B**) Immunohistochemical staining analysis of DICER1 protein in mouse epididymis. (**C**) The distribution of DICER1 protein in mouse epididymal cells. IS, initial segment; CAP, caput epididymis; COR, corpus epididymis; CAU, cauda epididymis.

the *Dicer1* mRNA was significantly downregulated in the same region (Figure 2F).

### Dicer1 deletion induces age-associated fertility defects

Macroscopic evaluation of epididymides in the 2 M *Dicer1*$^{-/-}$ mice revealed no obvious difference from those of wild-type mice (Figure 3A). The hematoxylin-eosin (HE) staining assay showed no obvious difference of cell layer and sperm (Figure 3B). Bromodeoxyuridine (5-bromo-2′-deoxyuridine, BrdU) can be incorporated into the newly synthesized DNA of replicating cells as an analog of thymidine and indicates the proliferative cells *in vivo*. The results of BrdU assay showed almost no cell underwent proliferation in the control and the *Dicer1*$^{-/-}$ group. Besides, the hochest staining showed no apoptotic cell both in the control and the *Dicer1*$^{-/-}$ tissue (Figure 3C).

Next, we tested the quality of sperm from the *Dicer1*$^{-/-}$ mice and the control mice. Neither the motility nor the progressive level of sperm was different at the age of 2 M and 6-month-old (6 M). However, the percentage of motile and progressive motility of sperm from 13-month-old (13 M) *Dicer1*$^{-/-}$ mice markedly reduced compared with control (Figure 3D). Subsequently, we performed the mating experiments to investigate the fertility of *Dicer1*$^{-/-}$ mice. There was no significant difference in the fertilization rate of sexually mature *Dicer1*$^{-/-}$ male mice compared with that of WT male mice (2–6 M). However, a higher incidence of fertilizing defects was observed in the middle-aged *Dicer1*$^{-/-}$ males (7–13 M) (Figure 3E).

Dicer1 disruption leads to the downregulation of β-defensin genes associated with antibacterial property

To panoramically understand the change of mRNAs by *Dicer1* knockout in CAP4/5, we performed the transcriptome sequencing (Figure 4A). We identified 15847 unique mRNAs that were mapped to the mouse transcriptome. Among them, about 77% mRNAs were unchanged, only 9% genes were downregulated and 14% genes were upregulated (Figure 4B). Interestingly enough, the highly expressed genes seemed more inclined to be downregulated upon *Dicer1* knockout (Supplementary Figure S1). Moreover, the highly expressed genes in the top, such as *β-defensin 30* (*Defb30*), *Bin1b*, and *β-defensin 17* (*Defb17*), were all the members of β-defensin gene family. The gene ontology (GO) pathway enrichment analysis showed that these downregulated genes were involved in the defense response to bacterium which was mainly responsible by β-defensin genes (Figure 4C). And the vast majority of β-defensin genes were downregulated upon *Dicer1* ablation (Figure 4D).

In order to confirm the changes of β-defensin genes, we picked up several β-defensin genes and performed quantitative analysis by qPCR. The results showed that the mRNA level of these β-defensin genes increased dramatically in 13 M mice. Interestingly, the expression of *Dicer1* also increased with age in epididymis. The expression of β-defensin genes decreased in the epididymis of 2 M *Dicer1*$^{-/-}$ mice, and the reduction of β-defensin gene expression was also identified in the CAP4/5 of 13 M *Dicer1*$^{-/-}$ mice (Figure 5A).
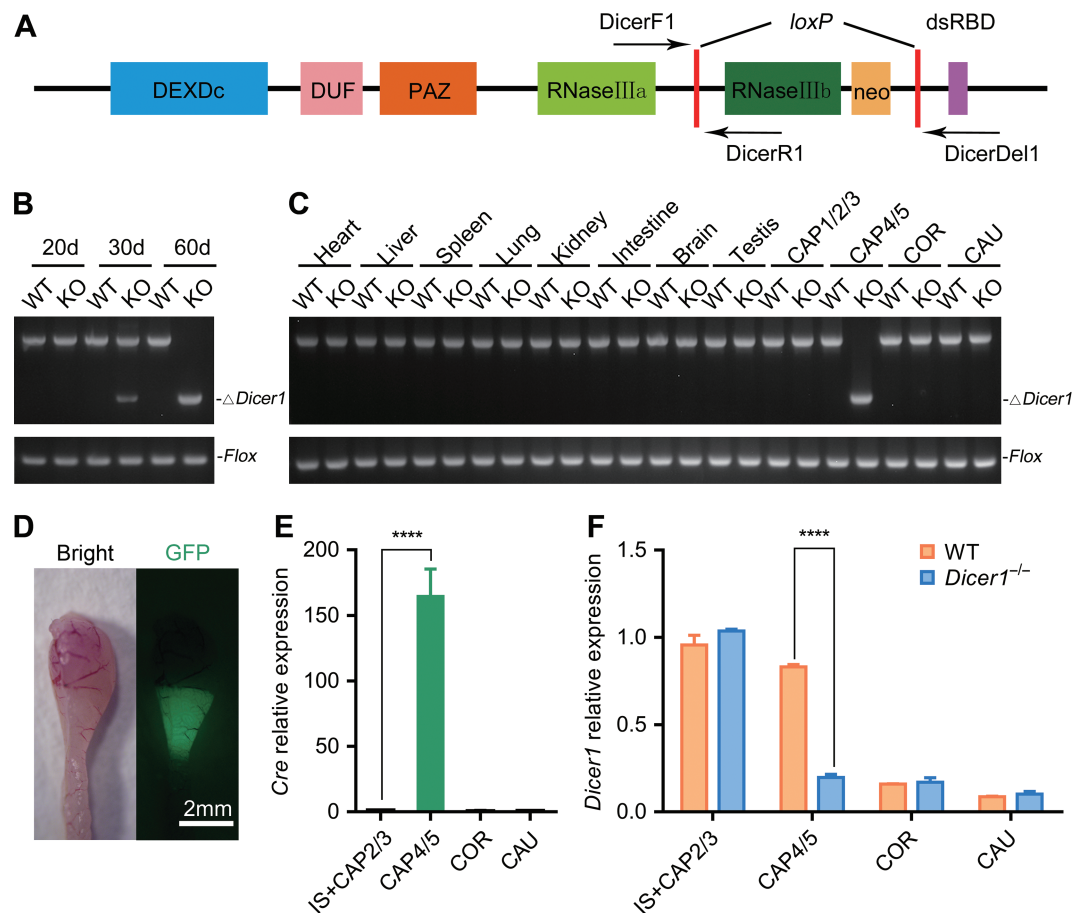
Figure 2 Generation of *Dicer1* conditional knockout mice. (**A**) The schematic diagram of the targeted *Dicer1* deletion strategy. The exon 23 that encodes most of the RNaseIIIb domain was flanked by two *loxP* sites. Black arrows show the location of genotyping primers used for detecting the floxed allele and the deletion allele. (**B**) The deletion of exon 23 started at Day 30 after birth in mouse epididymis and appeared stronger with development. (**C**) The deletion of exon 23 occurred specifically in caput epididymis. (**D**) *mT/mG* mouse was used as a tool for visualization of Lcn5-CRE activation. In the *Dicer1*$^{-/-}$ mouse, principle cells located in caput segment IV & V (CAP4/5) expressed the green fluorescent protein (GFP) with the action of CRE. (**E**) RT-qPCR analysis measuring the mRNA level of *Cre*. The *Cre* expression level was dramatically upregulated in CAP4/5 of *Dicer1*$^{-/-}$ mice compared with IS+CAP2/3 group (set as 1), and the location of CRE was consistent with the fluorescent image. Data in the bar graph are expressed as mean ± SEM ($n = 3$). ****$P < 0.0001$, one-way ANOVA followed by Bonferroni's multiple comparisons test. (**F**) RT-qPCR analysis measuring the mRNA level of *Dicer1* in epididymis. Compared to control mice, the expression of *Dicer1* was significantly downregulated in the region with CRE. The expression of *Dicer1* mRNA in IS+CAP2/3 group of WT mice is set as 1. Data in the bar graph are expressed as mean ± SEM ($n = 3$). ****$P < 0.0001$, two-way ANOVA, followed by Sidak's multiple comparisons test.

Furthermore, we singled out the *Bin1b* which expressed in CAP3/4/5 as a representative gene to investigate the change of β-defensin protein. The immunohistochemical staining assay showed a decreased level of BIN1B protein expression in the *Dicer1*$^{-/-}$ group (Figure 5C). β-defensin genes are androgen-dependent. Here, we did not find changes of the androgen receptor (AR) expression in the CAP4/5 (Figure 5B and D).

Escherichia coli and *Candida albicans* are the frequent pathogens of acute human epididymitis while *Staphylococcus aureus* is the seldom one (Michel et al., 2015). To verify the effect of β-defensins reduction on epididymal functions, we tested the antibacterial ability of CAP4/5 tissue homogenate using three different strains. The results indicated that the suppressive effect of tissue homogenate on *E.coli* dropped significantly in *Dicer1*$^{-/-}$ mouse (Figure 6A). Therewith, the result of *E.coli* challenge *in vivo* also showed the impaired antibacterial ability of *Dicer1*$^{-/-}$ epididymis. And the *E.coli* infection contributed to the inflammation of epididymis in *Dicer1*$^{-/-}$ group (Figure 6B and C). Actually, *Dicer1*$^{-/-}$ epididymis of 13 M mice showed swelling (Figure 6D) and retrograde infection (Supplementary Figure S2). And a large number of leukocyte cells were observed in the lumen gap of *Dicer1* KO epididymis (Figure 6E). Besides, epithelial damage and upregulated inflammatory cytokines including interleukin-1beta (*Il1b*), interleukin-6 (*Il6*) and tumor necrosis factor alpha (*Tnfa*) were present in *Dicer1* KO mice (Figure 6F).
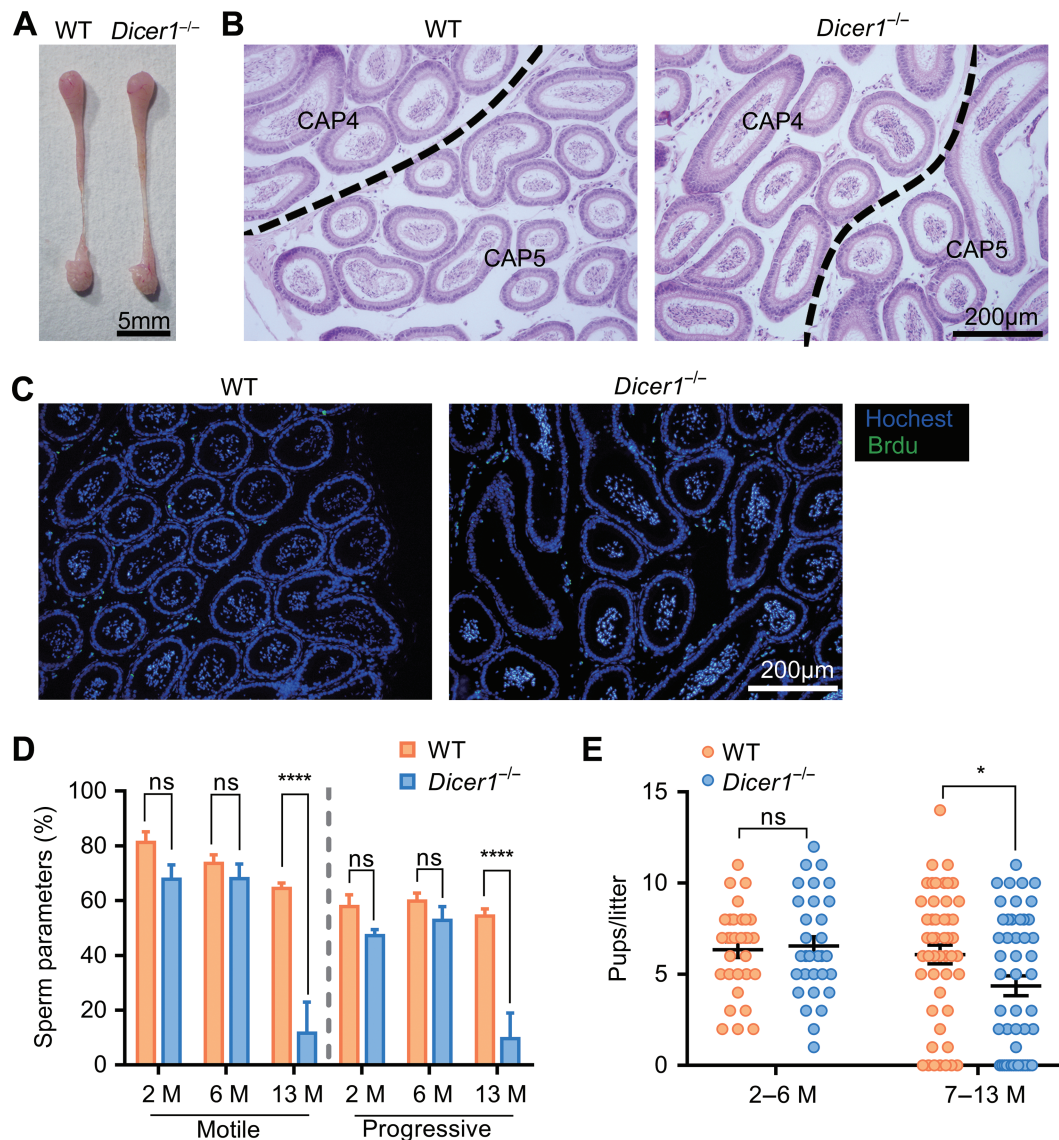
Figure 3 Morphology of the epididymis and fertility in $Dicer1^{-/-}$ mice. (**A**) The representative light microphotographs of epididymis in control mice and $Dicer1^{-/-}$ mice. (**B**) HE staining visualizing the histological structure of CAP4/5 in control mice and $Dicer1^{-/-}$ mice. (**C**) BrdU and hochest staining detecting the cell proliferation and apoptosis in epididymis. Cells with the ability to proliferate were labeled by green fluorescent (BrdU). Nuclei were labeled by blue fluorescent (Hoechst). (**D**) Computer-assisted sperm analysis (CASA) measuring the motility of the sperm. Two primary parameters, 'motile' and 'progressive', were used to characterize the sperm motility. Data in the bar graph are expressed as mean $\pm$ SEM ($n = 5$). ns, not significant, ****$P < 0.0001$. Each parameter is analyzed, respectively, using two-way ANOVA followed by Sidak's multiple comparisons test. (**E**) Mating test detecting the fertility of $Dicer1^{-/-}$ male mice. The horizontal lines indicate mean $\pm$ SEM ($n = 5$). ns, not significant; *$P < 0.05$ (Mann–Whitney test).

The transcription of β-defensin genes are regulated by DICER1 in a miRNA-independent manner

To search the mechanism underlying the change of β-defensin expression, we performed the small RNA sequencing and miRNA binding sites analysis. As expected, miRNAs were significantly decreased in $Dicer1^{-/-}$ tissue (Supplementary Figure S3). Strikingly, 24% detectable genes in mouse CAP4/5 contained no miRNA binding sites while 74% genes contained more than two miRNA binding sites by scanning the whole genome to search conserved miRNA target sites using TargetScanMouse

7.1. (Figure 7A). Another interesting result was that in addition to $Bin1b$ (two binding sites), β-defensin 25 ($Defb25$, two binding sites), β-defensin 36 ($Defb36$, two binding sites) and β-defensin 48 ($Defb48$, one binding site), β-defensin genes practically had no miRNA target site, while average target sites of whole genome were 45.44 (Figure 7B). We isolated and cultured the primary cells from the CAP4/5 of mouse and knocked down the expression of $Dicer1$ and $Drosha$ which initiated miRNA biogenesis at the upstream of $Dicer1$. The qPCR results showed that downregulation of β-defensin genes was associated with $Dicer1$
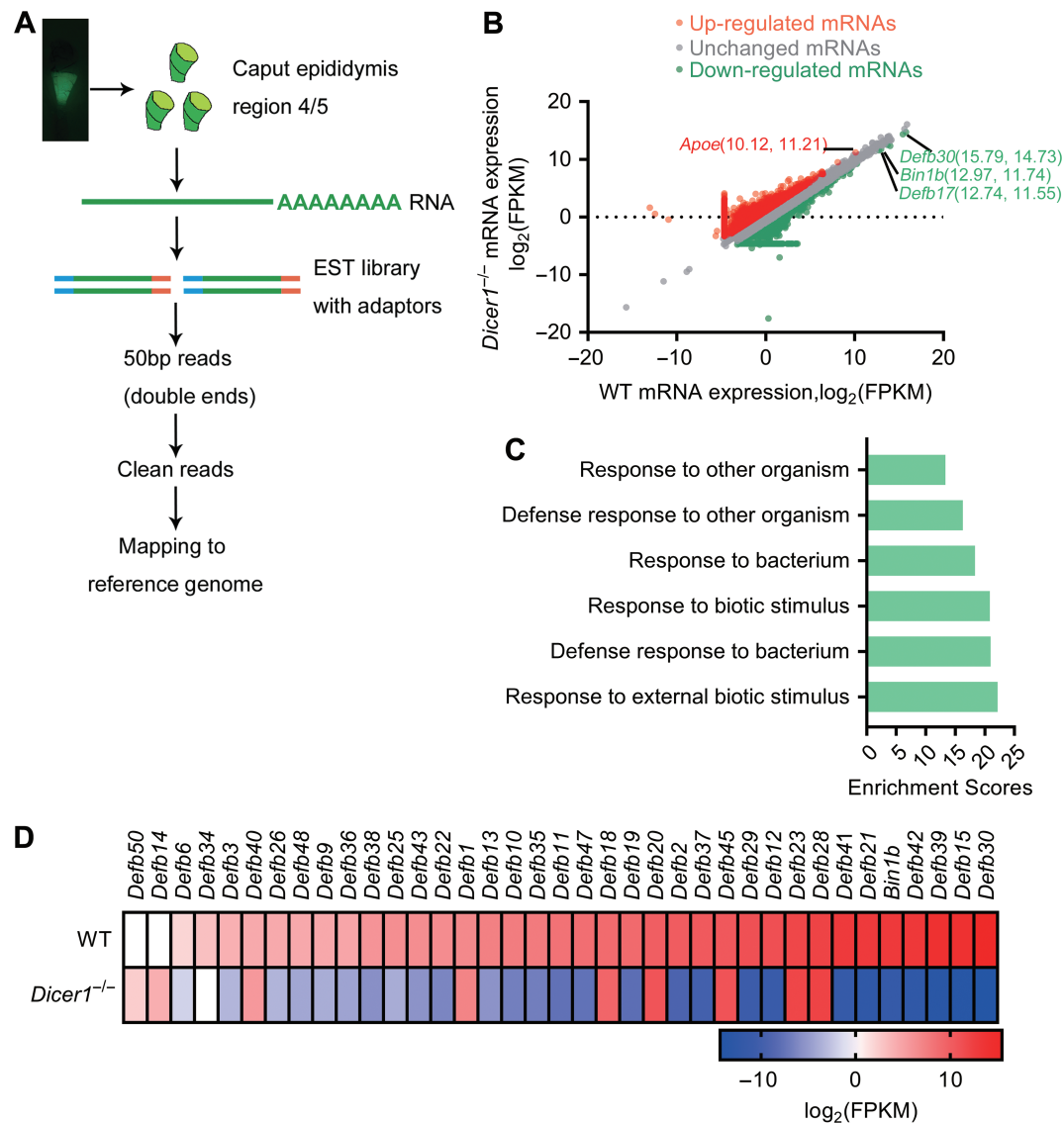
Figure 4 Comprehensive mapping of mRNAs in mouse CAP4/5. (**A**) Diagram of the workflow for constructing the library of transcriptome sequencing. (**B**) Differential expression profiles of genes in $Dicer1^{-/-}$ mice (fold change $\geq$2). (**C**) GO analysis of differential expression genes. (**D**) Heatmap of β-defensins profiles in control mice and $Dicer1^{-/-}$ mice.

rather than *Drosha* (Figure 7C). These results suggested that DICER1 could regulate the transcription of β-defensin genes with a miRNA-independent manner. Next, we found an enrichment of DICER1 in the promoter of *Bin1b* gene by ChIP-qPCR. And POL II failed to bind to the transcription start site (TSS) of *Bin1b* upon *Dicer1* knockout (Figure 7D). Moreover, the transcriptional activity of *Bin1b* declined in $Dicer1^{-/-}$ cells and wild-type DICER1 could rescue it (Figure 7E). The RNase III domains and PAZ domain of DICER1 are essential for miRNA biogenesis (Yuan et al., 2014). Double mutation of the RNase IIIa and IIIb (D1310A/D1693A) or mutation of the PAZ (Y961A/Y962A) resulted in failing to generate mature miRNAs (Supplementary Figure S4). The DICER1 with mutations still rescued the gene transcription (Figure 7E). These results revealed that DICER1 could exploit DNA to reach its targets and regulated the transcription of β-defensin genes (Figure 7F).

Discussion

The sperm maturational disorders were identified among up to 40% of infertile men (Cornwall, 2009). It is truly frightening that couples who desire a child turn to assisted reproductive techniques such as intracytoplasmic sperm injection using suboptimal sperm regardless of increased defects in offspring. The better acquainted with encounters of sperm in epididymis may drive an alternative therapy. In this study, we created a conditional knockout mouse line in which *Dicer1* was deleted in the principal cells of distal caput epididymis in adult mice to explore the role of epididymis in sperm maturation.
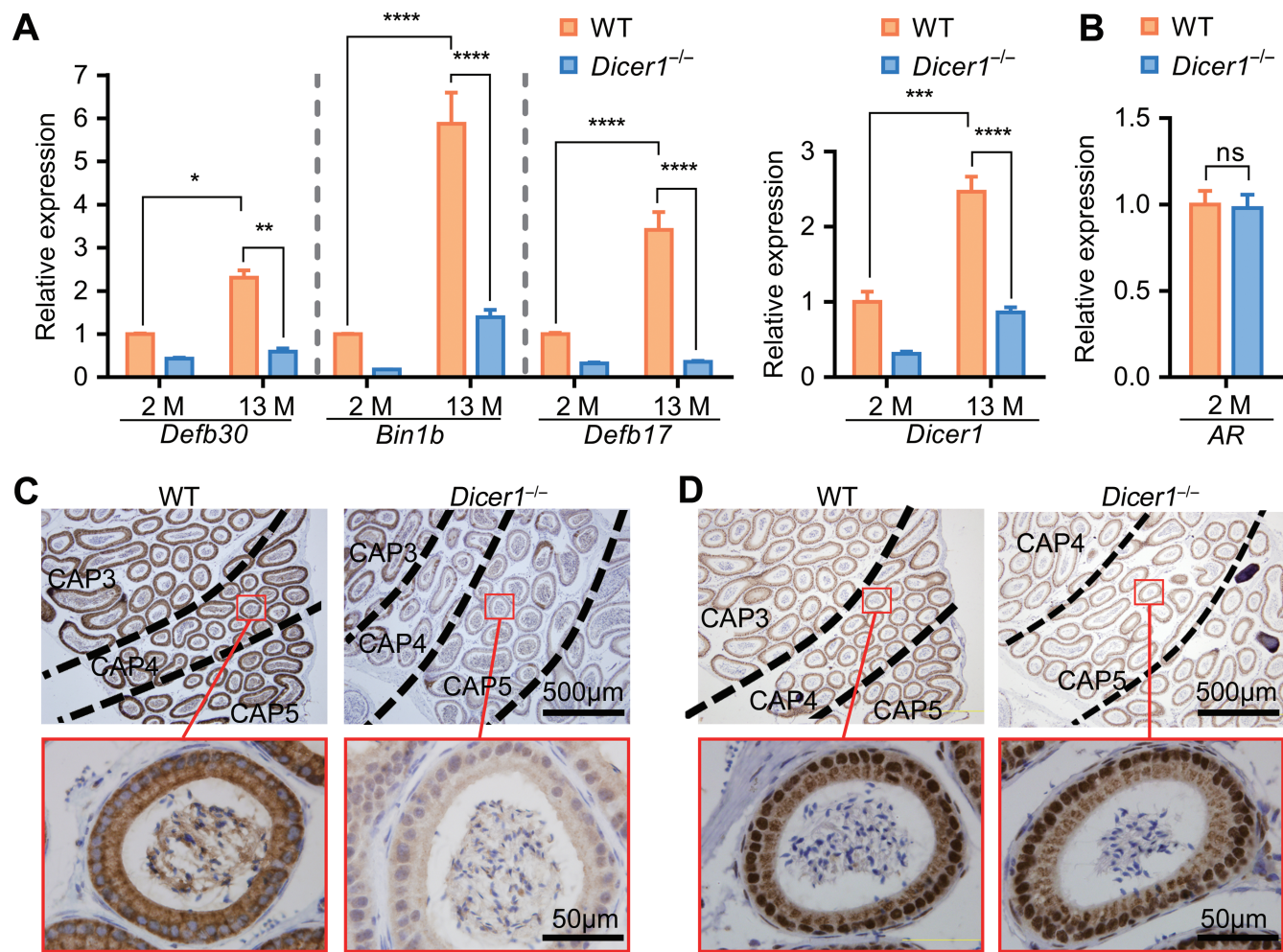
Figure 5 *Dicer1* deficiency leads to downregulation of β-defensin genes. (**A**) RT-qPCR analysis measuring the mRNA level of *Defb30*, *Bin1b*, *Defb17*, and *Dicer1* in *Dicer1*$^{-/-}$ epididymis. The expression of *Defb30* and *Dicer1* in 2 M WT mice is set as 1. Data in the bar graph are expressed as mean ± SEM ($n = 3$). ***$P < 0.001$, ****$P < 0.0001$. Each gene is analyzed respectively using two-way ANOVA followed by Tukey's multiple comparisons test. (**B**) RT-qPCR analysis measuring the mRNA level of *AR* in *Dicer1*$^{-/-}$ epididymis. The expression of *AR* in WT group is set as 1. Data in the bar graph are expressed as mean ± SEM ($n = 3$). ns, not significant, unpaired *t*-test. (**C**) Immunohistochemical staining analyzing the protein level of BIN1B in *Dicer1*$^{-/-}$ epididymis. (**D**) Immunohistochemical staining analyzing the protein level of AR in *Dicer1*$^{-/-}$ epididymis.

Consistent with previous studies, the biogenesis of miRNAs, especially the biogenesis of high expressed miRNAs, was disturbed obviously upon *Dicer1* ablation in the mouse epididymis. As the result of miRNA action, target mRNAs usually are silenced. So, the downregulation of miRNAs which is due to the deletion of *Dicer1* certainly increases the expression of target mRNAs. As expected, quite a number of the mRNAs were upregulated in the *Dicer1*$^{-/-}$ tissue. GO pathway analysis of differentially expressed mRNAs revealed that the deletion of *Dicer1* caused disturbance of antibacterial ability, which was associated with the β-defensin gene family. Interestingly, β-defensin genes were downregulated in the *Dicer1*$^{-/-}$ tissue. It may offer a clue to the new mode of gene regulation.

β-defensins are a set of secreted peptides involved in diverse processes including antibiosis, immune modulation, and sperm motility (Li et al., 2001; Diao et al., 2014; Dorin and Barratt, 2014). In the 2 M and 6 M *Dicer1*$^{-/-}$ mice, the sperm motility was not affected following the depletion of β-defensins. It could be that the depletion of β-defensins only existed in CAP4/5 while the expression in other regions was normal. But the ability of antibacteria was impaired significantly in the *Dicer1*$^{-/-}$ tissues both *in vitro* and *in vivo*. The phenotype of *Dicer1*-null epididymis looks like the human disease epididymitis extremely. The epididymitis is a common disease that may attack men at any age. And up to 40% of patients become sterile even after the cure (Rusz et al., 2012). In lipopolysaccharide- and microorganisms-induced epididymitis, the mRNA expression of β-defensins usually changes dramatically, which showed the relation of β-defensins and epididymitis (Michel et al., 2015). It is different from these models that our mouse model simulates
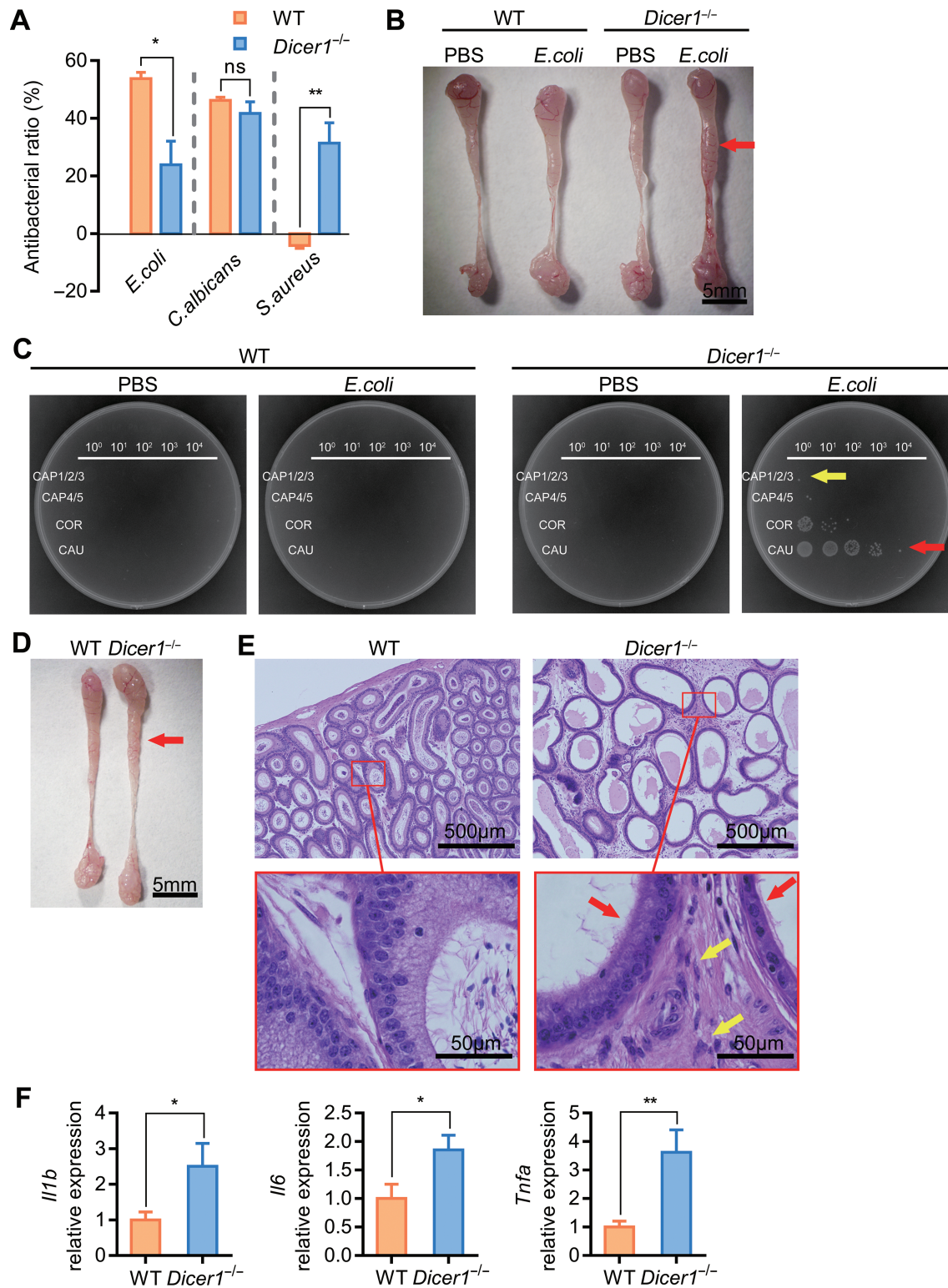
Figure 6 The expression of *Dicer1* is essential for the antibacterial properties of epididymis *in vitro* and *in vivo*. (**A**) Detection of the antimicrobial ability of *Dicer1*$^{-/-}$ epididymis *in vitro*. Data in the bar graph are expressed as mean ± SEM (*n* = 3). ns, not significant, *P < 0.05, **P < 0.01. The data of *E. coli* and *C.albicans* are analyzed, respectively, using unpaired *t*-test. The data of *S.aureus* are analyzed using unpaired *t*-test with Welch's correction. (**B**) Bacterial challenge assay measuring the antimicrobial ability of *Dicer1*$^{-/-}$ epididymis *in vivo*. *E. coli k12* suspension was injected in mouse epididymis from deferent duct to construct a simulate model of epididymis infection. The contralateral epididymis was injected with equal volume of PBS serving as the performance control. The epididymis of *Dicer1*$^{-/-}$ mice infected

the infection *in vivo* after the imbalance of homeostasis. Our data showed epididymis dysfunction lead by *Dicer1* defect did not include the ability of anti-*C. albicans*. But, oddly, the ability to fight *S. aureus* was greatly strengthened in *Dicer1*$^{-/-}$ mice. In another project of our lab, we found the ability of anti-*S. aureus* decreased in the epididymis of *Bin1b* overexpression mice (unpublished). This may indicate the inner link between *Bin1b* and *S. aureus*. Nevertheless, more evidence is needed to come to light the inner mechanism.

Further exploration detected the retrograde infection and inflammatory infiltrations in epididymis as the *Dicer1*$^{-/-}$ mice grew up and became older, displaying the state of epididymitis which is much closer to real illness events. The under reasons could be ascribed that a subset of target genes being regulated by DICER1, such as β-defensin genes, could help epididymis to response to accumulated environmental stimuli. A comparative study of epididymal genes expression in people of different age showed that 9 of 19 β-defensin genes were upregulated in aged human epididymides, compared with young adult group (change fold ≥1.5) (Zhang et al., 2010). The present qPCR results of β-defensin genes in mouse epididymis agreed with the data from this comparative study. These secretory defensin peptides expressed highly in aged epididymis may contribute to maintain the homeostasis of epididymis. Thus, dysfunction of β-defensins by *Dicer1* defect leads to the higher risk of infection. In contrast, overexpression of β-defensin or intravenous administration of β-defensin peptide can reverse the epididymitis in *E.coli*-infected animals (Fei et al., 2012; Biswas et al., 2015). It may be a creditable attempt that giving supplements of β-defensin peptides to patients.

By the time *Dicer1*-null male mice reached 13 M, sperm count in the caudal epididymis decreased dramatically (data not shown) and sperms from these mice showed very low motility. The continuous tracking data of mating test showed a higher incidence of fertilizing defects in middle-aged *Dicer1*-deficient males compared with WT males of the same age, in spite of the seasonal variation of birth rate (Reynolds et al., 2017). The male fertility disorder in middle-aged *Dicer1*$^{-/-}$ mice appears to be a secondary effect induced by sperm contamination. These findings are supported by some clinical studies. About 15% of male infertility cases are assigned to the infection of urinary tract including infection of the testis, epididymis and prostate (Henkel et al., 2006; Pellati et al., 2008; Domes et al., 2012). The clinical study based on 1256 infertile men shows that about 33.2% of semen samples are contaminated with bacteria. Out of the 246 semen samples included finally in the study, 50 samples (20.3%) show the presence of *E. coli*, and 67.4% of individuals with *E. coli* infection are infertile. The under reason is that bacterial contamination contributes to the apoptosis and necrosis of sperm, which leads to serious infertility (Moretti et al., 2009). Moreover, a study demonstrated that the decrease of some β-defensins by immunologic stimulant and testicular inflammation might aggravate the infertility (Cao et al., 2010). Even with antibiotic treatment, up to 40% have fertility problems as a result of the disease (Rusz et al., 2012). So it is very important to develop new therapeutic approaches that can solve the fertility problems.

Using comprehensive analysis of transcriptome map and genome-wide miRNA binding sites, we identified the β-defensin genes not controlled by miRNAs. Then the *Dicer1* and *Drosha* were separately knocked down in the primary cells of mice epididymal epithelial with RNA interference. Using qPCR assay, we were able to verify and validate that the β-defensin genes were regulated by DICER1 directly but not by miRNAs, no matter whether the β-defensin gene harbored miRNA binding sites or not. To further uncover the mechanism of gene regulation, we selected *Bin1b*, a member of β-defensin family. We performed ChIP, *Dicer1* mutation (leading to the biogenesis disturbance of miRNAs), and fluorescence assay and were able to prove that the *Bin1b* gene was transcribed directly by *Dicer1*. These results confirm that DICER1 could shape the antibacterial ability by transcriptional regulatory activity.

Overall, this study may provide a new tool for investigating the underlying reasons of epididymitis and brings to the forefront a miRNA-independent transcriptional regulation role of DICER1 in modulating the antibacterial property of epididymis and consequent male fertility.

Materials and methods
Experimental animals
To obtain mice with inactive DICER1 in the principal cells of the caput epididymis, *Dicer1*$^{f1/f1}$ mice were bred to the *Lcn5-Cre* mice strain which can express specially CRE enzyme in principle cells in the distal caput of epididymis. The *Cre* allele was genotyped by standard PCR with the primers tgCre F and tgCre R. The floxed allele and the deletion allele were genotyped according to the published article (Harfe et al., 2005). All primer sequences used in this study are available in Supplementary Table S1.

RNA isolation and RT-qPCR
For mRNA detection, total RNA from mouse epididymis was isolated with Trizol reagent (Invitrogen), 0.5 μg RNA was reverse transcribed to cDNA according to the user's manual of ReverTra Ace qPCR RT Master Mix with gDNA Remover kit (TOYOBO). Then

with *E.coli k12* became inflamed. (**C**) Bacteria in the epididymis of *Dicer1*$^{-/-}$ mice appendix multiply rapidly (the red arrow), causing the retrograde infection (yellow arrows). CAP1/2/3: IS+CAP2/3. (**D**) The *Dicer1*$^{-/-}$ mice have higher risks of epididymis infection with age. The epididymis of 13 M mice showed swelling (the red arrow). (**E**) Tuble edema and massive infiltration of leukocytes were observed in the epididymis of 13 M mice in *Dicer1*$^{-/-}$ group (yellow arrows). Besides, microvilli missing of principle cells was also observed in *Dicer1*$^{-/-}$ group, indicating epididymal epithelial damages (red arrows). (**F**) RT-qPCR analysis measuring the mRNA level of *Il1b*, *Il6* and *Tnfa* in the epididymis of 13 M mice. The mRNA expression in WT group is set as 1. Data in the bar graph are expressed as mean ± SEM ($n = 8$). ns, not significant, *$P < 0.05$, **$P < 0.01$, unpaired *t*-test.
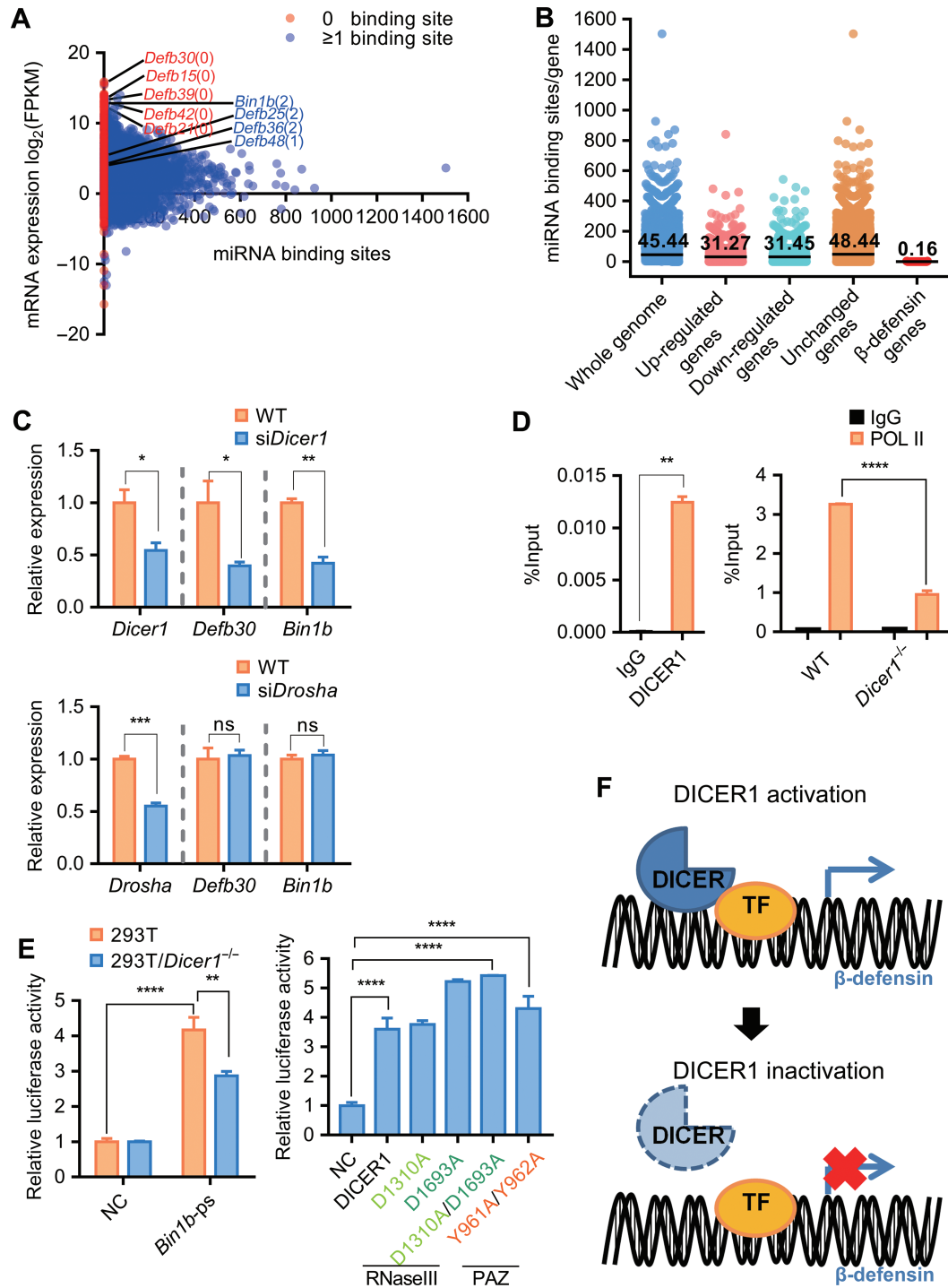
Figure 7 DICER1 regulates the transcription of β-defensin genes in a miRNA-independent way. (**A**) Conjoint analysis of mRNA expression and predicted miRNA binding sites. Y-axis shows the expression value of mRNAs ($\log_2$(FPKM)). X-axis shows the corresponding number of miRNA binding sites. This analysis reveals a set of genes that have 0 miRNA binding site, indicating that these genes are not regulated directly by miRNAs (red dots). (**B**) Analysis of average miRNA binding sites per mRNA. The short horizontal lines indicate the mean. (**C**) Contrastive analysis of the expression of defensin genes upon *Dicer1* knockdown and *Drosha* knockdown using RT-qPCR (mean ± SEM; $n = 3$). ns, not significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Each mRNA expression in WT group is set as 1 and is analyzed, respectively, using unpaired *t*-test. (**D**) Quantification of DICER1 and POL II in the promoter of *Bin1b* determined by ChIP (mean ± SEM; $n = 3$). **$P < 0.01$, paired *t*-test and two-way ANOVA followed by Sidak's multiple comparisons test. (**E**) Lucerferase assay testing the transcription activity of DICER1 (NC is set as 1, mean ± SEM; $n = 3$). **$P < 0.01$, ****$P < 0.0001$, ANOVA followed by Tukey's multiple comparisons test. (**F**) A model for transcription control of gene expression by DICER1.

qPCR was performed for quantification using standard procedures on a Rotor-Gene Q 5plex HRM Platform (QIGEN). For miRNA detection, RNA was extracted with mirVana miRNA Isolation Kit (Ambion), 0.5 µg RNA was reverse transcribed using the stem-loop RT primer as described (Kramer, 2011). Then the qPCR still was manipulated according to the standard procedures on a Rotor-Gene Q 5plex HRM Platform. All primer sequences used in this study are available in Supplementary Table S1.

Immunohistochemistry staining assay

Mouse epididymis was fixed in 4% paraformaldehyde and embedded in paraffin. After deparaffinage and rehydration, sections were dipped into 3% H_2O_2 for 15 min to block endogenous peroxidase. Then, these samples were boiled in citrate antigen retrieval solution twice, 5 min every time and naturally cooled to room temperature. For IHC, epididymis sections were incubated with BIN1B monoclonal antibody 1:50 (homemade in our lab), AR antibody 1:50 (ab74272, Abcam) in 10% (*v/v*) goat serum/PBS (ab7481, Abcam) overnight at 4°C. Sections were incubated with second antibody and colored with GTVision III Detection System/Mo&Rb (GK500705, GeneTech) followed by hematoxylin counterstaining.

BrdU incorporation assay

BrdU (B5002, Sigma-Aldrich) is diluted in PBS to make a sterile solution of 20 mg/ml. The BrdU solution was injected into mice abdominal cavity as concentration of 200 mg/kg. Then these animals were mercy killed using carbon dioxide 2 h after injection. The epididymis tissues were fixed with 4% paraformaldehyde for 24 h at 4°C, tissues were then dehydrated with the different concentration ethanol, vitrificated by dimethylbenzene, infiltrated with paraffin, and made of paraffin section. These samples were boiled in citrate antigen retrieval solution for 30 min after deparaffinage and cooled in ice water mixture immediately. For BrdU detection, these sections were incubated in 10% (*v/v*) goat serum/PBS at room temperature for 30 min and with the BrdU monoclonal antibody at 1:50 (05–633, Millipore) at 4°C overnight. After PBS washes three times for ~5 min per wash, the FITC-conjugated secondary antibody (Jackson Immuno Research) was added at 1:1000 at room temperature for 1 h. At last, sections were co-stained with Hochest 33258 (B2883, Sigma-Aldrich).

Fertility test

$Dicer1^{-/-}$ male mice and control mice were mated with C57BL/6 female mice last for a year. The physiological state and little size of mated female mice were closely observed during the experiment. And every mated female mouse was replaced by a new one after delivering two litters to avoid the effects from physiological changes of female mice.

Computer-assisted sperm analysis (CASA)

As previously described (Zhou et al., 2013), the epididymis was stripped away from the surrounding tissues such as fat-pad and blood vessels and so on. The cauda epididymis was cut and transferred into 1 ml EKRB medium which was warmed up to 37°C. To release the sperms sufficiently, cut the tissue in several places and incubate in medium for 5 min. Then the sperm was placed in a Sperm Analysis Chamber (Hamilton Thorne Research) and analyzed using a CASA machine (HTM-TOX IVOS sperm motility analyzer, Animal Motility, version 12.3 A; Hamilton Thorne Research) followed by concentration adjustment.

RNA-seq and analysis

For transcriptome sequencing, the total RNA was extracted from the segment IV & V of epididymis (CAP4/5), the poly-A containing mRNAs were enriched using oligo-dT attached magnetic beads from the total RNA. Then the mRNA library was constructed using TruSeq RNA Sample Preparation Kit V2 (Illumina) as follows: (i) Isolated mRNAs were fragmented into small pieces ~200 nt using divalent cations. (ii) Complementary DNAs (cDNAs) were synthesized. (iii) One microgram cDNA for each sample was used for end repair, A-tailing, and adapter ligation. (iv) The cDNAs with adapters were amplified using PCR. The library was amplified to produce clonal clusters on cBot (Illumina) using HiSeq PE Cluster Kit v4-cBot-HS (Illumina) and then was sequenced with HiSeq SBS Kit v4-HS (Illumina) by Illumina Hiseq 2000 (Illumina). Then, 100 bp paired-end reads were cleaned and mapped to mouse genome by Tophat2 short read mapper version 2.1.0. The differential expression genes were analyzed with fold change. GO analysis of differential expression genes (change fold ≥2) was performed by the Gene Ontology enRIchment anaLysis and visuaLizAtion tool (GORILLA, http://cbl-gorilla.cs.technion.ac.il/).

For small RNA-seq, 1 µg purified small RNA fragments were modified with 3′ adapter and 5′ adapter. Sequencing was performed using Illumina Hiseq 2000 followed reverse transcription and PCR amplification. The clean reads were mapped to the library of mouse miRNAs by Bowtie. The miRNA targets and corresponding annotations mRNAs were downloaded from TargetScanMouse (http://www.targetscan.org/mmu_71/mmu_71_data_download/ Conserved_Family_Conserved_Targets_Info.txt.zip).

Antimicrobial activity in vitro assay and bacterial challenge assay

Escherichia coli k12 strain (*E. coli k12*), candida albicans strain (*C.albicans*) and staphylococcus aureus strain (*S.aureus*) were preserved by our lab which was brought to 50% glycerol. The *E. coli k12* was grown in Luria-Bertani medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) with 50 mg/L streptomycin at 37°C for 4 h. The *C.albicans* was grown in Yeast extract-Trypton-Dextrose medium (2% trypton, 1% yeast extract, and 2% glucose) at 30°C for 4 h. The *S.aureus* was grown in Mueller–Hinton Broth (0.2% beef extract, 1.75% casein hydrolysate, and 0.15% starch) at 37°C for 4 h.

For antimicrobial activity *in vitro* assay, six pieces of epididymis segment IV & V from three mice were placed in a clean round-bottom microcentrifuge tube, and ice-cold PBS was added to the tube up to 300 µl. Then tissues were homogenized

rapidly on ice. *E. coli k12*, *C.albicans*, and *S.aureus* suspensions were washed with ice-cool PBS and the concentration of suspension was measured and adjusted to $1 \times 10^2/50$ µl, respectively. Tissue homogenate was divided into three parts, and then every part (100 µl/part) was mixed with 50 µl microbial solution and incubated on a plate medium with 1.5% agar at 37°C. The same volumn of PBS (100 µl) was incubated with 50 µl microbial solution as performance control. Colonies were counted after 16 h. The antimicrobial ratio was calculated according the formula as follow: antimicrobial ratio= $100\% \times$ (colonies[test]-colonies[performance_control])/ colonies[performance_control].

For bacterial challenge assay *in vivo*, the frozen *E. coli k12* was grown in LB with 50 mg/L streptomycin at 37°C for 4 h. The concentration of suspension was measured and adjusted. Then, 1×10^3 *E.coli k12* suspended in 5 µl sterile PBS was injected in mouse epididymis from deferent duct with a microinjection apparatus (NARISHIGE Group). The contralateral epididymis was injected, equal volume PBS serving as the performance control. The mice after surgery were sutured and housed for 3 days. Then the epididymis was dissected carefully and placed into a clean round-bottom microcentrifuge tube. Three pieces of tissues from three mice were homogenized rapidly on ice with 300 µl ice-cold PBS. Tissue homogenate was diluted gradiently and printed on the plate medium using a drop-plate method as described (Chen et al., 2003). Colonies were photographed and counted after 16 h.

Epididymal cell culture

The primary cells from mouse epididymis were cultured as described (Lin et al., 2002), with modifications. In brief, the epididymal tissues of region 4 and 5 were minced in PBS and then incubated in 1 mg/ml collagenase type IV (C5138, Sigma-Aldrich) for 2 h at 34°C in 5% CO_2. After incubation, fragments of epididymis were centrifuged to remove collagenase, transferred in 1 ml fresh IMDM medium (with 20% FBS, 1 nM DHT, and 10 µg/ml insulin) and separated to small cell clusters using pipette.

ChIP-qPCR

ChIP was performed as previously described (Hu et al., 2010), with modifications. In brief, epididymis region 4 and 5 tissues from three mice were pooled together and minced in PBS. Then, 4 µg ChIP Grade antibody to DICER1 (ab14601, Abcam) was used per ChIP reaction followed crosslinking and sonication. The protein A/G plus agarose beads (sc2003, Santa Cruz Biotechnology) were washed and DNA was eluted and purified. Then DNA was quantified by real-time quantitive PCR using standard three-step approach on a Rotor-Gene Q 5plex HRM Platform (QIGEN). The sequences of primer pairs DCR ChIP F, DCR ChIP R, POL II ChIP F and POL II ChIP R used in this study are available in Supplementary Table S1.

Construction of Dicer1 knockout 293T cell line

The gRNAs were designed using the sgRNAcas9 (Xie et al., 2014). Then the gRNA oligos were inserted into the PGL3-U6-sgRNA-PGK-puromycin vector and co-transfected 293T cells with pST1374-N-NLS-flag-linker-Cas9 plasmid. After 24 h, cells were incubated in medium with 1 µg/ml puromycin (Merck) and 10 µg/ml blasticidin (Sigma) for 72 h. Then cells were dissociated and planted in 96-well plate (one cell per well). The final surviving clones were analyzed by sequencing. The sequences of gRNAs hDCR_gRNAa5 F, hDCR_gRNAa5 R, hDCR_gRNAs10 F and hDCR_gRNAs10 R are available in Supplementary Table S1.

Statistical analyses

Statistical analyses were performed by using GraphPad Prism (version 7.00; GraphPad Software). *P*-values are indicated in the figures, and differences were considered significant when $P < 0.05$.

Author contributions: C.T. performed experiments, made data analysis, and wrote manuscript. M.N. performed experiments. S.X. and Yao Zhang performed sequencing data analysis. C.Z. and Z.N. performed animal surgeries. C.C. and L.W. performed data analysis. Yuchuan Zhou performed part of CASA experiments and edited the manuscript. Yonglian Zhang conceived of the study and edited the manuscript.

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