

# The RNase III Enzyme DROSHA Is Essential for MicroRNA Production and Spermatogenesis<sup>\*[5]</sup>

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Qiuxia Wu<sup>‡</sup>, Rui Song<sup>‡</sup>, Nicole Ortogero<sup>‡</sup>, Huili Zheng<sup>‡</sup>, Ryan Evanoff<sup>§</sup>, Chris L. Small<sup>§</sup>, Michael D. Griswold<sup>§</sup>, Satoshi H. Namekawa<sup>¶||</sup>, Helene Royo<sup>\*\*</sup>, James M. Turner<sup>\*\*</sup>, and Wei Yan<sup>‡1</sup>

From the <sup>‡</sup>Department of Physiology and Cell Biology, University of Nevada School of Medicine, Reno, Nevada 89557, the <sup>§</sup>School of Molecular Biosciences, Washington State University, Pullman, Washington 99164, the <sup>¶</sup>Division of Reproductive Sciences, Perinatal Institute, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio 45229, the <sup>||</sup>Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45229, and the <sup>\*\*</sup>Division of Stem Cell Biology and Developmental Genetics, Medical Research Council, National Institute for Medical Research, London NW7 1AA, United Kingdom

**Background:** miRNA biogenesis requires two RNase III enzymes, DROSHA and DICER.

**Results:** Lack of DROSHA in the male germ line leads to deficiency in miRNA production and male infertility.

**Conclusion:** DROSHA and DICER have both common and unique functions in male germ cell development.

**Significance:** This study reveals an essential role of DROSHA, DICER, and DROSHA-/DICER-dependent small noncoding RNAs spermatogenesis.

DROSHA is a nuclear RNase III enzyme responsible for cleaving primary microRNAs (miRNAs) into precursor miRNAs and thus is essential for the biogenesis of canonical miRNAs. DICER is a cytoplasmic RNase III enzyme that not only cleaves precursor miRNAs to produce mature miRNAs but also dissects naturally formed/synthetic double-stranded RNAs to generate small interfering RNAs (siRNAs). To investigate the role of canonical miRNA and/or endogenous siRNA production in spermatogenesis, we generated *Drosha* or *Dicer* conditional knock-out (cKO) mouse lines by inactivating *Drosha* or *Dicer* exclusively in spermatogenic cells in postnatal testes using the Cre-loxp strategy. Both *Drosha* and *Dicer* cKO males were infertile due to disrupted spermatogenesis characterized by depletion of spermatocytes and spermatids leading to oligoteratzoospermia or azoospermia. The developmental course of spermatogenic disruptions was similar at morphological levels between *Drosha* and *Dicer* cKO males, but *Drosha* cKO testes appeared to be more severe in spermatogenic disruptions than *Dicer* cKO testes. Microarray analyses revealed transcriptomic differences between *Drosha*- and *Dicer*-null pachytene spermatocytes or round spermatids. Although levels of sex-linked mRNAs were mildly elevated, meiotic sex chromosome inactivation appeared to have occurred normally. Our data demonstrate that unlike DICER, which is required for the biogenesis of several small RNA species, DROSHA is essential mainly for the canonical miRNA production, and DROSHA-mediated miRNA production is essential for normal spermatogenesis and male fertility.

chromosomal reduction, and haploid differentiation and eventually become male gametes called spermatozoa/sperm in the testis (1–3). Although spermatogenesis can be divided into mitotic, meiotic, and haploid/spermiogenesis phases based upon the three major cellular events, these processes actually occur concurrently within the seminiferous epithelium, and specific cellular associations are formed among developing male germ cells and between germ cells and their supporting somatic cells (e.g. Sertoli cells and peritubular myoid cells) (1, 2). These complex and highly regulated cellular processes require multilayered regulatory networks, which have been shown to involve regulators that function at both transcriptional (e.g. transcription factors, epigenetic modulators, large noncoding RNAs, etc.) and post-transcriptional (small noncoding RNAs, RNA-binding proteins, etc.) levels (4–10). Among the small noncoding RNAs (sncRNAs)<sup>2</sup> identified to date, miRNAs have been demonstrated to play a role as post-transcriptional regulators through binding to the 3'-untranslated regions (3'UTRs) of mRNAs and thereby affecting mRNA stability and translational efficiency (11–13).

The canonical miRNA biogenesis pathway has largely been defined, and it involves the processing of miRNA primary transcripts (i.e. primary miRNAs (pri-miRNAs)) into precursor miRNAs (pre-miRNAs) by the microprocessor complex consisting of mainly DROSHA, an RNase III enzyme, and its cofactor DGCR8 (also called PASHA) in the nucleus (14, 15). Exportin 5 then exports pre-miRNAs to the cytoplasm, where DICER, another RNase III enzyme, further cleaves the pre-miRNAs to produce two mature miRNAs. Mature miRNAs serve as sequence guides by directing their associated effector com-

Spermatogenesis refers to the process through which male germ line stem cells undergo mitotic multiplication, meiotic

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<sup>1</sup> To whom correspondence should be addressed: Dept. of Physiology and Cell Biology, University of Nevada School of Medicine, CMM Bldg., Rm. 202, 1664 North Virginia St., MS0575, Reno, NV 89557. Tel.: 775-784-7765; Fax: 775-784-6903; E-mail: wyan@unr.edu.

<sup>2</sup> The abbreviations used are: sncRNA, small noncoding RNA; miRNA, microRNA; pre-miRNA, precursor miRNA; pri-miRNA, primary miRNA; cKO, conditional knock-out; qPCR, quantitative PCR; ANOVA, analysis of variance; miRNA-Seq, miRNA deep sequencing; EGFP, enhanced GFP; MSCI, meiotic sex chromosome inactivation; P, postnatal day; E, embryonic day; endo-siRNA, endogenous siRNA; mG, membrane-bound EGFP; RPKM, reads/kb of exon model/million; mT, membrane-bound Tomato Red; TE, transposable element; IAP, intracisternal A particle element.

plexes (e.g. RNA-induced silencing complex) to their targets, which are usually located in the 3'UTRs of mRNAs, and exert effects that can be stabilization/destabilization of mRNAs or activation/suppression of translation, depending on the cellular context and functional status (16–19). DROSHA recognizes pri-miRNAs, which are long primary transcripts derived from RNA polymerase II-mediated transcription from a miRNA locus or loci of several miRNA genes forming a cluster, and contains one or multiple stem-loop regions formed by sequences of future mature miRNAs (20, 21). Unlike DROSHA, DICER not only cleaves stem-loop structures in pre-miRNAs but also dissects double-stranded RNAs (dsRNAs), either exogenous synthetic ones introduced into the cell or naturally occurring ones in the cell, into small RNAs, such as mature miRNAs, small interfering RNAs (siRNAs), and endo-siRNAs (22, 23). Therefore, ablation of *Dicer* affects the production of all DICER-dependent small RNAs, whereas loss of *Drosha* or *Dgcr8* affects largely the formation of pre-miRNAs and consequently mature miRNA production (20, 21).

Inactivation of *Dicer* in mice leads to embryonic lethality at about embryonic day 7.5 (E7.5) (24), suggesting an essential role in post-implantation embryonic development. Conditional knock-out (cKO) of *Dicer* in various organs or cell lineages has revealed that DICER is required for normal development and function of almost every single cell type or organ tested so far (25–45), suggesting an essential role of DICER-dependent sncRNAs in normal physiology of the cell or organs. Ablation of DGCR8, a cofactor of DROSHA essential for the RNase III activity of DROSHA, results in embryonic lethality in ~E6.5, implying an essential role of miRNAs in early embryonic development (46). By comparing the phenotypes of cKO mice deficient in *Dicer* or *Dgcr8* in developing oocytes, two studies discovered that it is endo-siRNAs, but not miRNAs that are essential for normal oocyte development and maturation, and miRNA functions in developing oocytes are largely suppressed (47, 48). These data suggest that although both DICER and DROSHA are involved in miRNA biogenesis, the effects of ablation of DICER or DROSHA can be different because they control the production of different species of sncRNAs. Thus, a comparative study of *Dicer* and *Drosha* cKO mice may reveal phenotypes unique to either *Dicer* or *Drosha* inactivation.

Several previous studies have reported an essential role of DICER in primordial germ cell development and spermatogenesis by analyzing cKO mice with *Dicer* inactivation in the male germ line at different developmental time points (49–53). However, given that *Dicer* ablation simultaneously eliminates multiple sncRNA species (46–48, 54), the phenotype cannot be ascribed solely to miRNAs. To evaluate the specific role of miRNAs, germ line-specific inactivation of *Drosha* or *Dgcr8* is required. In addition, one common problem associated with the Cre-loxp strategy in generating cKO mice is the incomplete penetrance of Cre expression/activity in the targeted cell type, which usually leads to hypomorphism and mosaicism (55–57). To overcome this problem, we generated two compound cKO mouse lines, in which *Drosha* or *Dicer* was specifically inactivated in postnatal male germ cells, and meanwhile, the Cre-expressing male germ cells were labeled with membrane-bound EGFP (mG) as a reporter to monitor true *Drosha*-null or *Dicer*-

null spermatogenic cells *in vivo*. This study was designed with the following goals: 1) to define the physiological role of *Drosha* in miRNA biogenesis and in spermatogenesis *in vivo*; 2) to compare potential phenotypic differences between *Drosha* and *Dicer* cKO males because any difference, if observed, should reflect the potential roles of endo-siRNAs as *Drosha* cKO cells lack only canonical miRNAs, and *Dicer* cKO cells are deficient in both canonical miRNAs and endo-siRNAs; and 3) to determine transcriptomic changes in purified *Drosha*-null or *Dicer*-null spermatogenic cells, which have never been performed thus far. Here, we report our findings.

## EXPERIMENTAL PROCEDURES

**Generation of Postnatal Male Germ Line-specific *Drosha* or *Dicer* Knock-out Mice**—All of the animal work performed was approved by the Institutional Animal Use and Care Committee (IACUC) of the University of Nevada, Reno. *Dicer*<sup>lox/lox</sup> mice (The Jackson Laboratory) (30) and *Drosha*<sup>lox/lox</sup> mice (58) were bred with *Stra8-iCre* mice (The Jackson Laboratory) (59) to generate *Stra8-iCre-Dicer*<sup>+/lox</sup> and *Stra8-iCre-Drosha*<sup>+/lox</sup> offspring. These heterozygotes were further crossed with *Dicer*<sup>lox/lox</sup> and *Drosha*<sup>lox/lox</sup> mice to obtain *Stra8-iCre-Dicer*<sup>lox/lox</sup> and *Stra8-iCre-Drosha*<sup>lox/lox</sup> males. *Rosa26mTmG* mice (The Jackson Laboratory) (60) were used as a Cre reporter line to visualize the Cre-expressing cells. Female *Stra8-iCre-Dicer*<sup>lox/lox</sup> and *Stra8-iCre-Drosha*<sup>lox/lox</sup> were crossed with *Dicer*<sup>lox/lox</sup>-*Rosa26mTmG*<sup>tg/tg</sup> and *Drosha*<sup>lox/lox</sup>-*Rosa26mTmG*<sup>tg/tg</sup> males, respectively, to produce *Stra8-iCre-Dicer*<sup>lox/lox</sup>-*Rosa26mTmG*<sup>+/tg</sup> and *Stra8-iCre-Drosha*<sup>lox/lox</sup>-*Rosa26mTmG*<sup>+/tg</sup> offspring for morphological analyses, spermatogenic cell purification, and subsequent molecular analyses.

**Histological and Immunohistochemical Analyses**—Testes were dissected and fixed in Bouin's solution overnight at 4 °C followed by paraffin embedding. Paraffin sections (5 μm) were prepared and stained with the periodic-acid Schiff (PAS) solution (Sigma) for histological analyses. For observing membrane-bound Tomato Red (mT) and EGFP (mG) in control and cKO testes and for immunofluorescent analyses, cryosections were prepared. Testes from control (*Stra8-iCre-Rosa26mTmG*<sup>+/tg</sup>), *Drosha* cKO (*Stra8-iCre-Drosha*<sup>lox/lox</sup>-*Rosa26mTmG*<sup>+/tg</sup>), and *Dicer* cKO (*Stra8-iCre-Dicer*<sup>lox/lox</sup>-*Rosa26mTmG*<sup>+/tg</sup>) were dissected and fixed in 4% paraformaldehyde in PBS (pH 7.4) for 2 h at room temperature. The fixed testes were then incubated with serial sucrose solutions with increasing concentration from 5 to 20% followed by incubation in 20% sucrose in PBS overnight at 4 °C. The testes were then embedded in OCT and 20% sucrose mixture with a volume ratio of 1:1. Cryosections of 10 μm were either stained with DAPI to visualize the cell nuclei for morphological analyses or subjected to immunofluorescent analyses. Cryosections were mounted using the Aqua mounting medium (Fisher) and stored in dark at 4 °C for imaging analyses. Images were captured using confocal laser scanning system (Olympus, FV1000).

**TUNEL Assay**—Paraffin testis sections were used for TUNEL analyses of apoptotic cells. TUNEL was performed using the ApoTag Plus Peroxidase In Situ Apoptosis Detection kit (Millipore, Billerica, MA), according to the manufacturer's instructions.

**Purification of Pachytene Spermatocytes and Round Spermatids from Adult Control and cKO Testes**—Pachytene spermatocytes and round spermatids were purified from control (*Stra8-iCre-Rosha26mTmG<sup>+/tg</sup>*) and cKO (*Stra8-iCre-Drosha<sup>lox/lox</sup>-Rosha26mTmG<sup>+/tg</sup>* and *Stra8-iCre-Dicer<sup>lox/lox</sup>-Rosha26mTmG<sup>+/tg</sup>*) adult testes using a mini-STA-PUT method modified from the method described previously (61, 62).

**Small and Large RNA Isolation**—mirVana<sup>TM</sup> miRNA isolation kit (Ambion, Grand Island, NY) was used to isolate both large and small RNA fractions from purified pachytene spermatocytes and round spermatids following the manufacturer's protocol.

**Western Blot Analyses**—Protein isolation and Western blot analyses were performed as described previously (63). GAPDH was used as a loading control, and the rabbit anti-GAPDH polyclonal antibody was purchased from Cell Signaling Technology (catalog no. T4026). The rabbit anti-DROSHA polyclonal antibody was also from Cell Signaling Technology (catalog no. 3364). The rabbit anti-DICER polyclonal antibodies were generated by GenScript Inc. according to our antigen design, and its specificity has been evaluated (54).

**miRNA Deep Sequencing (miRNA-Seq)**—Small RNA cDNA libraries were prepared using the Ion Total RNA-Seq kit (Invitrogen). Briefly, small RNAs were ligated to an ion adaptor sequence. A complementary reverse transcription primer was used to anneal the ion adaptor to initiate reverse transcription and generate cDNAs using ArrayScript reverse transcriptase. Small RNA cDNAs were subsequently size-selected using the Novex TBE/urea gel system (Invitrogen) and amplified using AmpliTaq DNA polymerase. Emulsion PCR was used to clonally amplify cDNA on ion sphere particles. This was done using the Ion OneTouch (Invitrogen) in conjunction with the Ion OneTouch Template kit (Invitrogen). Enrichment for templated ion spheres was performed using the Ion OneTouch Enrichment System (Invitrogen) and Dynabeads MyOne Streptavidin C1 beads (Invitrogen). Subsequent sequencing of templated ion sphere particles was performed on an Ion Torrent Personal Genome Sequencer (Invitrogen) using the Ion Sequencing kit Version 2.0 (Invitrogen) and Ion 314 chips.

Sequencing reads were aligned using the Ion Torrent Server t-map aligner (Invitrogen). Aligned reads were analyzed using Partek Genomic Suite (version 6.6 beta, Partek, St. Louis, MO). To begin, aligned reads were matched to known mature miRNA sequences downloaded from the Functional RNA Database (fRNAdb) (64). To account for variances in sequencing depth and transcript length, the abundance of miRNA reads was normalized using a modified reads/kb of exon model/million (RPKM) (65). RPKM = total miRNA reads/(total mapped reads (million)  $\times$  miRNA sequence length (kb)). Transcripts with low abundance ( $<1$  RPKM) in control and cKO samples were filtered and removed from expression analyses. RPKM values were normalized to the mean expression for generating heat maps showing differential expression analysis.

**Large RNA cDNA Synthesis and qPCR**—After large RNAs, including total mRNAs, were isolated using the mirVana<sup>TM</sup> miRNA isolation kit (Ambion), DNA contamination was removed by DNase treatment using DNA-free<sup>TM</sup> DNase (Ambion). After DNase treatment, 25  $\mu$ l of large RNA was

mixed with 2  $\mu$ l of 10 mM dNTP and 1  $\mu$ l of random primers (3  $\mu$ g/ $\mu$ l, Invitrogen). The mixture was heated at 65 °C for 5 min and incubated on ice for 1 min. 8  $\mu$ l of 5 $\times$  First-Strand Buffer, 2  $\mu$ l of 0.1 M DTT, 1  $\mu$ l of RNase OUT (Invitrogen), and 1  $\mu$ l of SuperScript III reverse transcriptase (Invitrogen) were added to the mixture and incubated at 50 °C for 60 min. 40  $\mu$ l of H<sub>2</sub>O was added to the mixture; the concentration of the large RNA cDNAs was measured, and then they were diluted into 25 ng/ $\mu$ l as PCR working templates. By using gene-specific forward and reverse primers (supplemental Table S1), SYBR Green-based real time quantitative PCR was performed to examine mRNA expression levels. *Gapdh* or  $\beta$ -actin was used as an internal control. IAP, LINE1, and SINE B2 primer sequences are listed in supplemental Table S1.

**Microarray Analysis**—Large RNAs, including total mRNAs, were isolated from purified pachytene spermatocytes and round spermatids of control (*Stra8-iCre-Rosha26mTmG<sup>+/tg</sup>*) and cKO (*Stra8-iCre-Drosha<sup>lox/lox</sup>-Rosha26mTmG<sup>+/tg</sup>* and *Stra8-iCre-Dicer<sup>lox/lox</sup>-Rosha26mTmG<sup>+/tg</sup>*) testes using the mirVana<sup>TM</sup> miRNA isolation kit (Ambion), and DNA contamination was removed using DNA-free<sup>TM</sup> DNase (Ambion). RNA purity was examined by measuring  $A_{260/280}$  ratios using a spectrophotometer (NanoDrop, Wilmington, DE), and RNA quality was analyzed using the Bioanalyzer (Agilent Technologies, Santa Clara, CA). Each sample was prepared in two biological replicates. These RNA samples were then subject to labeling, hybridization, washing, data acquisition, and analyses as described previously (66).

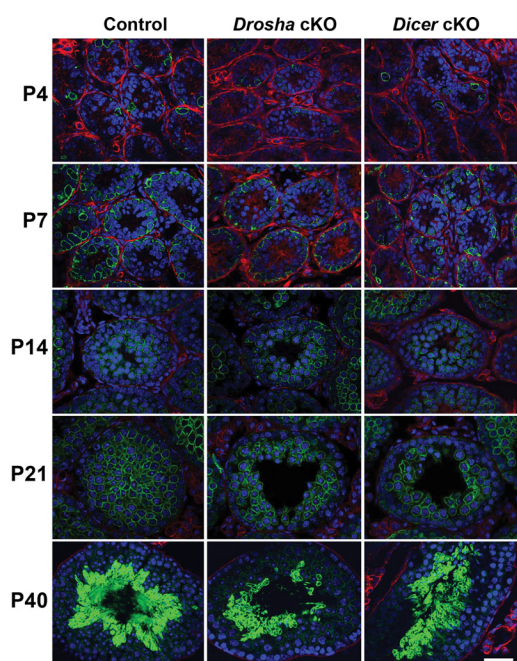
**RNA FISH**—RNA FISH analyses of *de novo* transcription of a Y-linked gene *Uty* was performed as described previously (67, 68). Cot-1 RNA FISH in conjunction with immunofluorescent staining was carried out following a published protocol (69).

## RESULTS

**Generation of Postnatal Male Germ Cell-specific *Drosha* or *Dicer* Conditional Knock-out Mice**—Global inactivation of either *Dgcr8* (46) or *Dicer* (24) leads to embryonic lethality, precluding functional analyses of postnatal germ cell development. To specifically inactivate *Drosha* or *Dicer* in postnatal male germ cells, we crossed a *Drosha-loxp* (*Drosha<sup>lox/lox</sup>*) (58) or a *Dicer-loxp* (*Dicer<sup>lox/lox</sup>*) (30) line with a *Stra8* (stimulated by retinoic acid gene 8)-*iCre* transgenic line (59), which has been shown to express improved Cre (iCre) recombinase exclusively in male germ cells with an initial expression in the differentiating spermatogonia at postnatal day 3 (P3) (59). The *Drosha* conditional allele has two loxp sites flanking exon 9, and the excision of exon 9 induced by iCRE recombination would lead to frameshift and result in multiple stop codons in exon 11 (58). The *Dicer* conditional allele contains two loxp sites flanking exon 23 of *Dicer*, which encodes for the most part the second RNase III domain (30), and thus iCRE-mediated recombination would inactivate DICER cleavage activity. It has been validated that after iCre-mediated recombination, the activity of DROSHA or DICER is completely lost (30, 58).

To visualize the iCre-mediated germ cell-specific deletion of *Drosha* or *Dicer*, *Stra8-iCre-Drosha<sup>lox/lox</sup>* and *Stra8-iCre-Dicer<sup>lox/lox</sup>* mice (herein called *Dicer* or *Drosha* cKO mice, respectively) were further crossed with a global double-fluores-





**FIGURE 1. Germ cell-specific ablation of *Drosha* or *Dicer* in postnatal testes.** Cross-sections of seminiferous tubules of control (*Stra8-iCre-Rosa26mTmG<sup>+/tg</sup>*), *Drosha* cKO (*Stra8-iCre-Drosha<sup>lox/lox</sup>-Rosa26mTmG<sup>+/tg</sup>*), and *Dicer* cKO (*Stra8-iCre-Dicer<sup>lox/lox</sup>-Rosa26mTmG<sup>+/tg</sup>*) testes at P4, P7, P14, P21, and P40. Expression of membrane-bound EGFP (mG) is indicative of Cre expression and activity. mG was first detected in some of the spermatogonia in P4 testes. The number of mG-positive germ cells increased with testicular development. In P21 testes, the highest levels of mG were observed in spermatocytes and round spermatids. In P40, mG was detected in almost all spermatogenic cells, but the highest levels were observed in the cytoplasmic membrane of elongated spermatids. Note that prior to P21, not all germ cells were mG-positive. Bars, 20  $\mu$ m.

cent Cre reporter mouse line *Rosa26mTmG<sup>tg/tg</sup>* (60) to generate *Drosha* and *Dicer* compound knock-out mice (*Stra8-iCre-Drosha<sup>lox/lox</sup>-Rosa26mTmG<sup>+/tg</sup>* and *Stra8-iCre-Dicer<sup>lox/lox</sup>-Rosa26mTmG<sup>+/tg</sup>*, herein called *Drosha* and *Dicer* reporter cKO mice, respectively). In these compound knock-out mice, cells without Cre activity expressed membrane-targeted tandem dimer Tomato Red fluorescent protein (mT), whereas cells with Cre activity had the loxp-flanked *mT* cassette deleted, thus allowing the expression of a downstream membrane-targeted EGFP (*mG*) cassette. Therefore, Cre-expressing cells showed green fluorescence on the cytoplasmic membrane, representing the true knock-out cells. By introducing the fluorescent reporter allele into the *Drosha* or *Dicer* cKO background, we could determine the penetrance of Cre expression and activity, monitor the morphological changes of “true” KO cells, and purify the KO cells for molecular analyses.

The membrane-bound EGFP (mG) was first observed in spermatogonia in the testes of control, *Drosha*, and *Dicer* reporter cKO mice at about P4 (Fig. 1), which is consistent with a previous report showing the initial expression in differentiating spermatogonia at P3 in the *Stra8-iCre* mouse line (59). It is noteworthy that only a small proportion of spermatogonia displayed mG expression at P4, and the number of mG-positive spermatogenic cells increased from P7 to P21 (Fig. 1). By P40, the highest levels of mG were observed in the cell membrane of elongated spermatids, and the intensity of mG was higher in round spermatids and spermatocytes than in spermatogonia

(Fig. 1). The fact that almost all of the spermatocytes and round spermatids at P21 and P40 were mG-positive is consistent with the previous report showing iCre expression starts in type A spermatogonia and ends in preleptotene spermatocyte (59). Based upon the spatiotemporal expression patterns of mG, we conclude that the iCre-mediated *Drosha* or *Dicer* deletion occurred to a full penetrance by P21, and in adult cKO testes, at least all pachytene spermatocytes and haploid germ cells were truly *Drosha*- or *Dicer*-null.

**Inactivation of *Drosha* or *Dicer* in Postnatal Spermatogenic Cells**—We purified pachytene spermatocytes and round spermatids using the STA-PUT method (62, 70, 71), and purity of the two cell types was  $\sim 90\%$  based upon counting of mG (green) versus mT (red) cells (Fig. 2). Using purified pachytene spermatocytes and round spermatids, we performed qPCR to examine levels of *Drosha* or *Dicer* mRNAs. Levels of *Drosha* and *Dicer* mRNAs (corresponding to the portion encoded by the floxed exons) were significantly reduced in the *Drosha*- and *Dicer*-null pachytene spermatocytes and round spermatids, respectively (Fig. 3, A and B). Given that the purity of the cells was  $\sim 90\%$ , it is highly likely that the remaining levels were derived from other contaminating testicular cell types. *Drosha* mRNA levels in the two *Dicer*-null spermatogenic cell types remained unchanged, whereas levels of *Dicer* mRNAs in *Drosha*-null pachytene spermatocytes and round spermatids were slightly elevated (Fig. 3, A and B). Previous studies have shown that iCRE-mediated deletion of the floxed *Dicer* and *Drosha* alleles would result in a mutant form of DICER without the second RNase III domain and a total loss of DROSHA due to nonsense mRNA decay, respectively (30, 58). To further confirm the expression of DICER and DROSHA protein in these cKO spermatogenic cells, we also performed Western blot analyses (Fig. 3C). Consistent with previous studies (30, 58), reduced levels of the slightly truncated DICER protein were detected in *Dicer* cKO pachytene spermatocytes, whereas DROSHA protein was mostly absent in *Drosha* cKO pachytene spermatocytes (Fig. 3C). Levels of DICER and DROSHA in *Drosha* and *Dicer* cKO cells were unchanged, respectively. These results were consistent with their respective mRNA levels (Fig. 3, A and B). These data further support the findings of the mTmG reporter assays *in vivo* (Fig. 1), demonstrating that a close to full penetrance of the Cre activity could be reached in pachytene spermatocytes and their subsequent cell type, round spermatids, in the two cKO mouse lines.

A previous report suggests that DROSHA may be involved in the 45 S pre-rRNA processing pathway, and DROSHA inactivation may thus affect the production of 18 S and 28 S rRNAs, leading to translational defects and cell death (72). To examine whether DROSHA deficiency can lead to defective production of 18 S and 28 S rRNAs *in vivo*, we analyzed levels of 18 S and 28 S rRNAs in control, *Drosha*, and *Dicer* cKO pachytene spermatocytes and round spermatids using qPCR (Fig. 3, D and E). No significant changes in levels of 18 S and 28 S rRNAs, as well as their precursor 45 S rRNAs, were observed among control, *Drosha*, and *Dicer* cKO pachytene spermatocytes (Fig. 3D). In round spermatids, both 18 S and 28 S rRNA levels showed no changes, whereas 45 S rRNA levels were slightly up-regulated (Fig. 3E). These data suggest that a lack of DROSHA does not

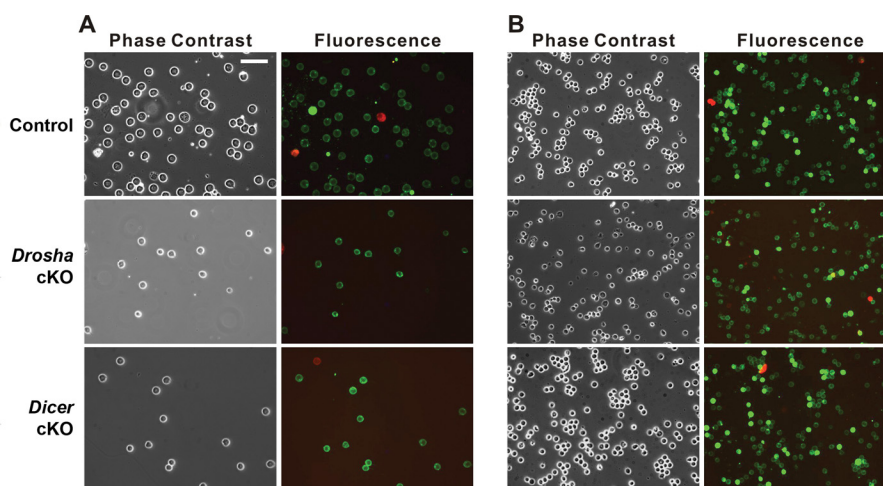


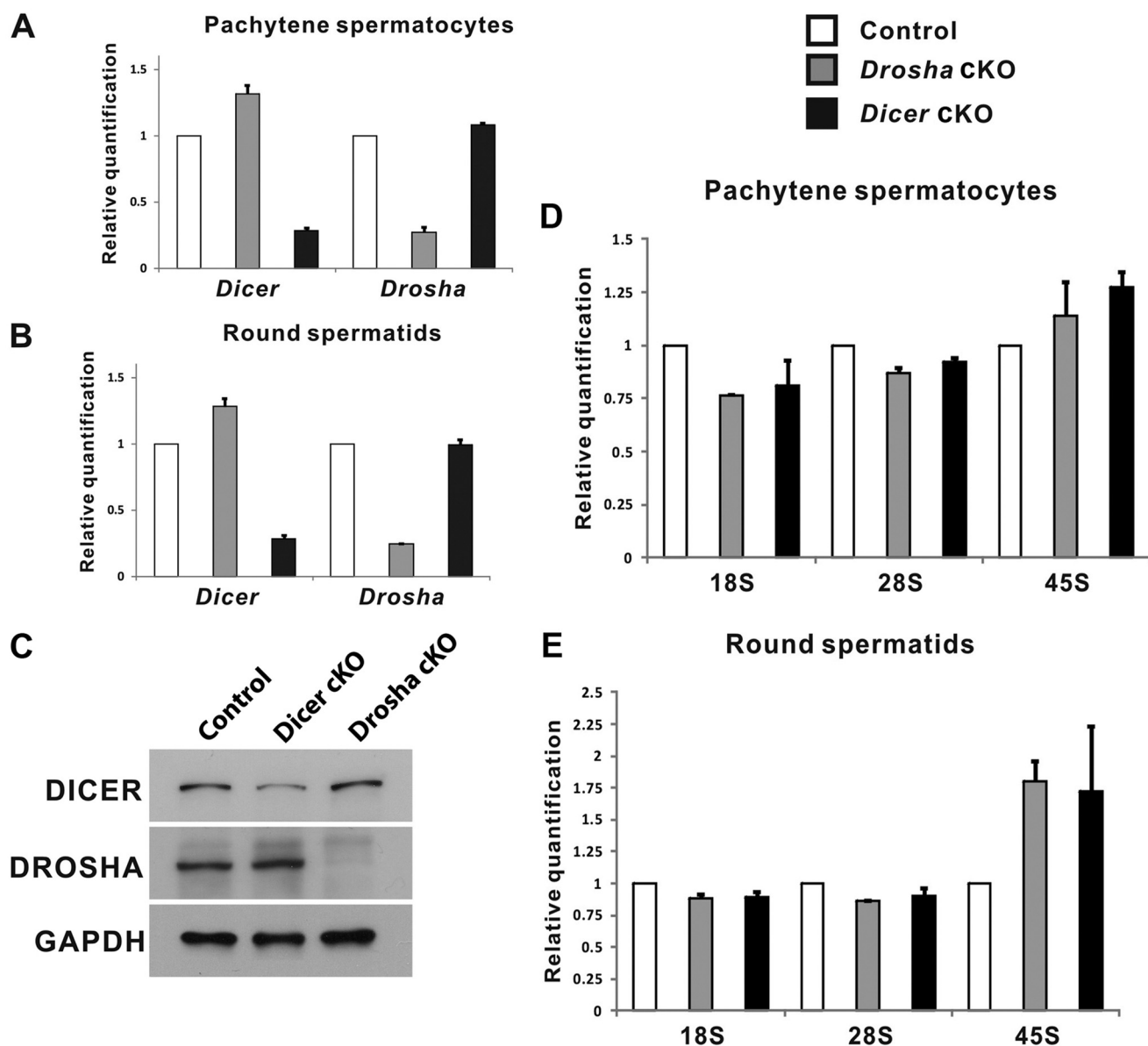
FIGURE 2. **Purification of pachytene spermatocytes and round spermatids from control, *Drosha*, and *Dicer* cKO testes.** A, phase-contrast and fluorescent images of purified pachytene spermatocytes. B, phase-contrast and fluorescent images of purified round spermatids. Green cells are the Cre-expressing pachytene spermatocytes or round spermatids, and the rare red cells represent non-Cre-expressing spermatogonia or somatic cells (i.e. Sertoli or Leydig cells). All panels are in the same magnification. Bar, 20  $\mu$ m.

significantly affect 18 S/28 S rRNA production. This finding is consistent with an earlier report showing DGCR8 inactivation does not affect the 45 S rRNA processing pathway (46).

**DROSHA Is Required for miRNA Biogenesis, whereas DICER Is Essential for the Production of Both miRNAs and Endo-siRNAs**—We recently identified numerous endo-siRNAs in the mouse testes (54). Using the same *Dicer* and *Drosha* cKO mice, we demonstrated that the biosynthesis of testicular endo-siRNAs requires DICER activity but is independent of DROSHA activity (54). To determine the effects of *Dicer* or *Drosha* ablation in spermatogenic cells, we performed miRNA-Seq using the Ion Torrent Personal Genome Sequencer (Fig. 4). A total of 213 and 223 unique mature miRNA sequences were identified in pachytene spermatocyte and round spermatids, respectively (supplemental Table S2). Levels of 123 miRNAs identified from pachytene spermatocytes and 110 miRNAs from round spermatids with high abundance in controls were presented as heat maps (Fig. 4). ~80% of all miRNA species detected displayed decreased levels in both *Dicer* and *Drosha* cKO cells, whereas ~1/3 was completely absent in cKO cells (Fig. 4 and supplemental Table S2). Interestingly, several miRNAs had increased levels in cKO cells compared with controls, and those increased in *Dicer* cKO cells were either absent or down-regulated in *Drosha* cKO cells and vice versa. The ones up-regulated in *Dicer* cKO cells but absent or down-regulated in *Drosha* cKO cells may represent those noncanonical miRNAs that have been mis-categorized in the fRNAdb (64). Similarly, those up-regulated in *Drosha* cKO cells, but absent or down-regulated in *Dicer* cKO cells, should be endo-siRNAs. More thorough bioinformatic and experimental validation assays are needed in the future to assign the correct identity for those miRNAs. Nevertheless, to our knowledge, this was the first miRNA-Seq assay on *Dicer* or *Drosha* cKO spermatogenic cells, and our results are consistent with and supportive of the current concept on DICER and DROSHA activities and the biogenesis of miRNAs and endo-siRNAs (73). Based upon the difference in DICER and DROSHA activities, phenotypic variations observed between the *Dicer* and *Drosha* cKO mice should in theory be

ascribed to endo-siRNAs because endo-siRNA production in *Drosha*-null germ cells is unaffected, whereas miRNAs are absent in both *Drosha*- and *Dicer*-null male germ cells.

***Drosha* or *Dicer* cKO Mice Display Severe Disruptions in Both Meiotic and Haploid Phases of Spermatogenesis**—Fertility tests by breeding *Drosha* or *Dicer* cKO males with WT adult females indicated that neither *Drosha* nor *Dicer* cKO males were fertile despite their normal mating behavior. To investigate the cause of infertility in *Drosha* or *Dicer* cKO male mice, we performed morphological analyses on *Drosha* or *Dicer* cKO testes at both gross (Fig. 5, A and B) and light microscopic (Fig. 5, C and D) levels. The adult *Drosha* or *Dicer* cKO testes were much smaller in size (Fig. 5A) than control testes of their heterozygous littermates (*Stra8-iCre-Drosha*<sup>+/lox</sup> and *Stra8-iCre-Dicer*<sup>+/lox</sup>). By analyzing the testis weight during testicular development (Fig. 5B), a significant decrease in testis weight was initially observed at P21 in both *Drosha* and *Dicer* cKO males, and by P40, the cKO testes weighed ~50% of the control testes. Consistent with the reduced testis weight, examination of the testis histology revealed severe germ cell depletion in both adult *Drosha* (Fig. 5C) and *Dicer* (Fig. 5D) cKO testes. Although littermate controls displayed robust spermatogenesis (Fig. 5, C, panel a, and D, panel a), both *Drosha* and *Dicer* cKO testes showed severely disrupted seminiferous epithelia containing few or no elongated spermatids (Fig. 5, C, panel b, and D, panel b). The presence of numerous vacuoles (arrows in panels c and d in Fig. 5, C and D) and multinucleated “giant” cells (arrowheads in panel d in Fig. 5, C and D) was indicative of active spermatogenic cell depletion. Accordingly, the control epididymis contained numerous spermatozoa (Fig. 5, C, panel e, and D, panel e), whereas the cKO epididymides were largely devoid of spermatozoa (Fig. 5, C, panel f, and D, panel f). The spermatogenic cells that were depleted or being depleted were mainly spermatocytes and early spermatids in both cKO testes. Interestingly, *Drosha* cKO testes appeared to be more severely disrupted than *Dicer* cKO testes at least at histological levels (Fig. 5, C, panel b, and D, panel b) because a lot more spermatogenic cells were present in *Dicer* cKO testes than in *Drosha* cKO testes. To



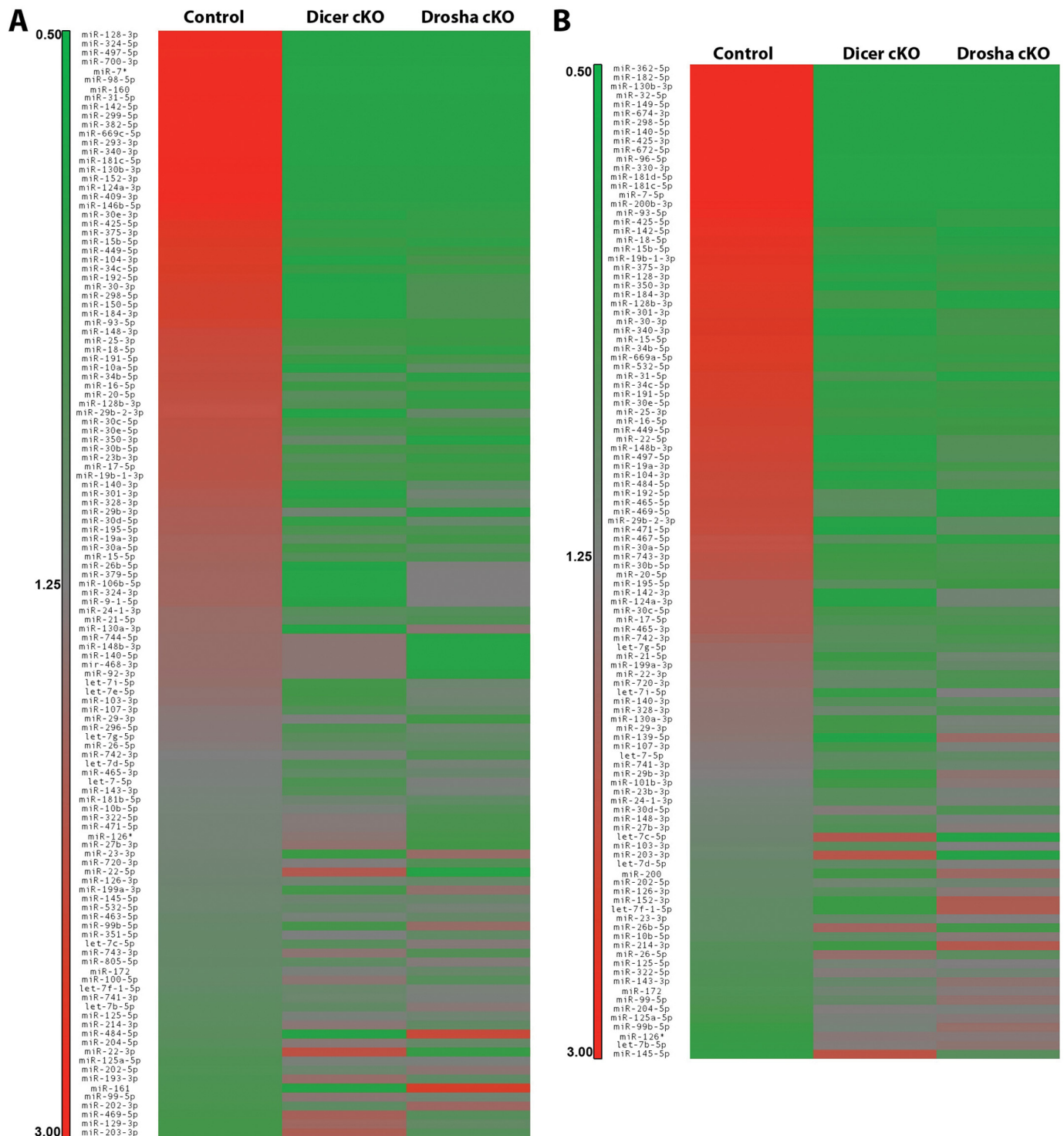
**FIGURE 3. Levels of *Drosha* or *Dicer* mRNA and protein as well as 18 S, 28 S, and 45 S ribosomal RNAs in pachytene spermatocytes and round spermatids purified from control (*Stra8-iCre-Rosa26mTmG<sup>+/tg</sup>*), *Drosha* cKO (*Stra8-iCre-Drosha<sup>lox/lox</sup>-Rosa26mTmG<sup>+/tg</sup>*), and *Dicer* cKO (*Stra8-iCre-Dicer<sup>lox/lox</sup>-Rosa26mTmG<sup>+/tg</sup>*) testes.** A, qPCR analyses of *Drosha* and *Dicer* mRNA levels in *Drosha* or *Dicer* cKO pachytene spermatocytes. B, qPCR analyses of *Drosha* and *Dicer* mRNA levels in *Drosha* or *Dicer* cKO round spermatids. C, Western blot analyses of levels of DICER and DROSHA in control (wild type), *Dicer*, or *Drosha* cKO pachytene spermatocytes. D, qPCR analyses of levels of 18 S, 28 S, and 45 S rRNAs in control, *Drosha*, or *Dicer* cKO pachytene spermatocytes. E, qPCR analyses of levels of 18 S, 28 S, and 45 S rRNAs in control, *Drosha*, or *Dicer* cKO round spermatids.

quantify the phenotypic differences between *Drosha* and *Dicer* cKO testes, we analyzed the proportions of seminiferous tubule cross-sections that were devoid of elongating/elongated spermatids (Fig. 5E) or contained vacuoles/multinucleated giant cells (Fig. 5F). In control testes, almost all tubule cross-sections contained either elongating (steps 9–11) or elongated (steps 12–16) spermatids. In contrast, ~23 and ~7% of tubule cross-sections in *Drosha* and *Dicer* cKO testes were devoid of elongating/elongated spermatids, respectively (Fig. 5E). Moreover, vacuoles or multinucleated giant cells were observed in only ~1% of the control tubule cross-sections, whereas ~43% and ~24% of the tubule cross-sections in *Drosha* and *Dicer* cKO testes contained numerous vacuoles and/or multinucleated cells, respectively (Fig. 5F). These data suggest that *Drosha* cKO

testes displayed more severe spermatogenic cell depletion than *Dicer* cKO testes. Nevertheless, the depleted spermatogenic cell types were mainly spermatocytes and spermatids in both cKO testes, suggesting an essential role for either *Drosha* or *Dicer* to support the meiotic and haploid phases of spermatogenesis in mice.

Consistent with partial Cre penetrance in P4 and P7 cKO testes (Fig. 1), no discernable histological changes were observed in either *Drosha* or *Dicer* cKO testes between P4 and P12 (data not shown). By P14, histological changes in the seminiferous epithelial structures became discernable in both *Drosha* and *Dicer* cKO testes (Fig. 6A). Mid-pachytene spermatocytes were less in number, and some displayed cytoplasmic shrinkage with highly condensed nuclei resembling struc-





**FIGURE 4. Absent or severely reduced miRNA expression in *Dicer* or *Drosha* cKO spermatogenic cells revealed by miRNA-Seq analyses.** *A*, heat map representing levels of miRNAs with a 2-fold up- or down-regulation among control (wild type), *Dicer*, and *Drosha* cKO pachytene spermatocytes. *B*, heat map representing levels of miRNAs with a 2-fold up- or down-regulation among control (wild type), *Dicer*, and *Drosha* cKO round spermatids. Original miRNA-Seq data can be found in supplemental Table S2.

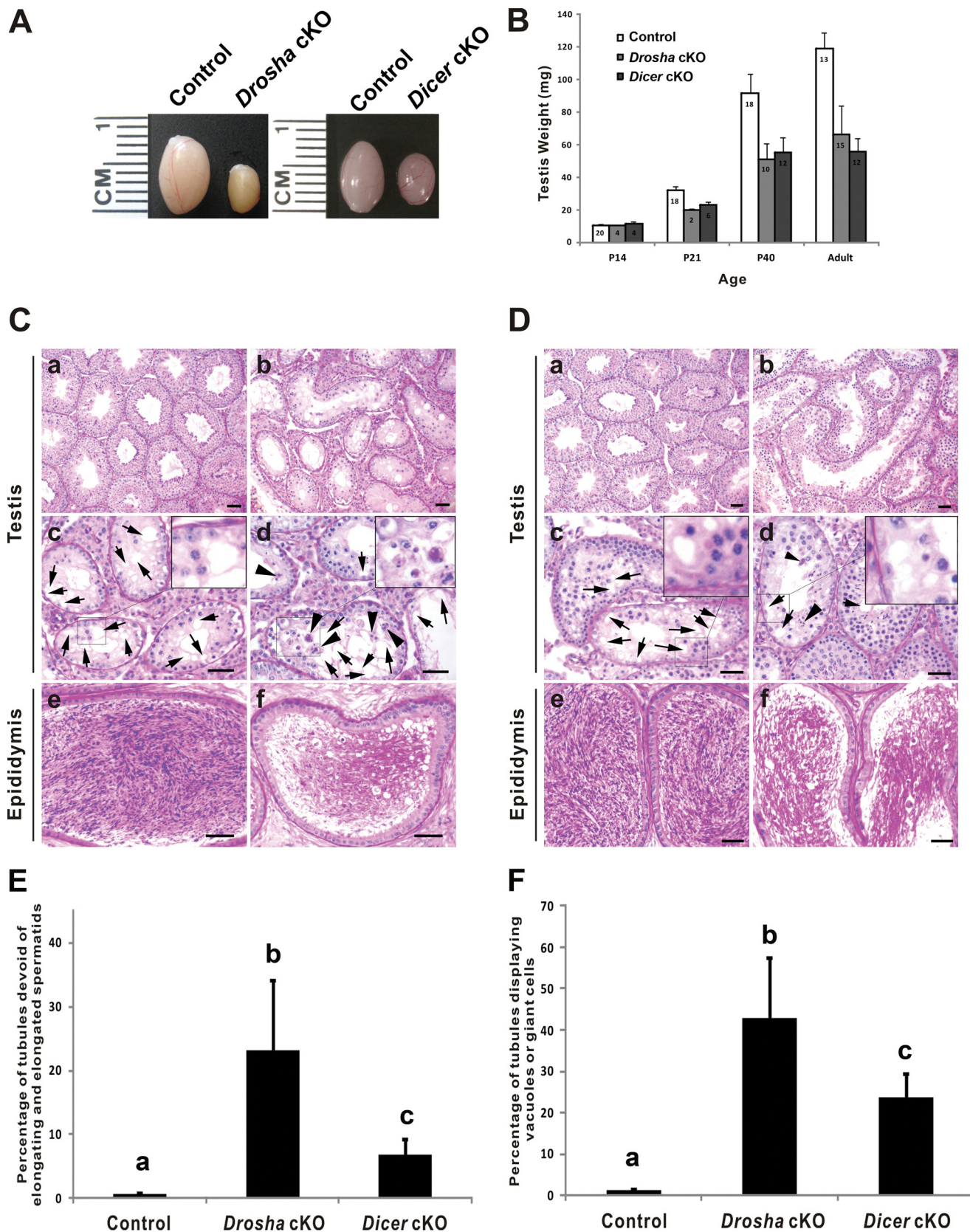
tural features of early apoptotic cells. Depletion of pachytene spermatocytes became more and more obvious/severe afterward, and the severity of disruptions reached to the maximum at P28 (Fig. 6A). At P40, although the germ cell depletion was still ongoing, the rate of depletion appeared to decrease because seminiferous epithelia of both cKO testes contained all types of

spermatogenic cells despite a lower number compared with controls. TUNEL assays revealed enhanced apoptosis in spermatocytes in both *Drosha* and *Dicer* cKO testes (Fig. 6B). Interestingly, round spermatids that were being depleted were TUNEL-negative, supporting the notion that massive depletion of round spermatids is mainly achieved through detaching from

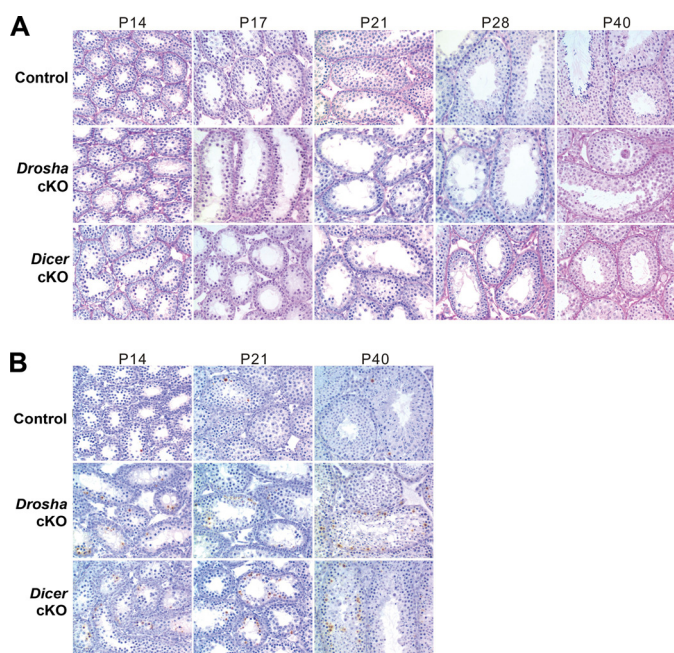
# Male Germ Line Ablation of Drosha Impairs Spermatogenesis

the seminiferous epithelium and sloughing into the lumen instead of the classic apoptotic pathway (74–76). Histological analyses of the developing testes (Fig. 6) further sup-

ported the observation in the adult cKO testes (Fig. 5), suggesting the spermatogenic cell types that were depleted were mainly pachytene spermatocytes and spermatids, and the







**FIGURE 6. Onset of germ cell depletion during testicular development in *Drosha* or *Dicer* cKO mice.** A, Bouin's solution-fixed, paraffin-embedded cross-sections of the control (heterozygous littermates), *Drosha* (*Stra8-iCre-Drosha<sup>lox/lox</sup>*), and *Dicer* (*Stra8-iCre-Dicer<sup>lox/lox</sup>*) cKO testes were stained with the PAS reagent. Germ cell depletion was first detected at P14 and became more and more severe between P17 and P28 in both *Drosha* and *Dicer* cKO testes. B, TUNEL assays of apoptotic germ cells in control, *Drosha*, or *Dicer* cKO testes at P14, P21, and P40. Note that enhanced apoptosis was mainly observed in spermatocytes (brown staining).

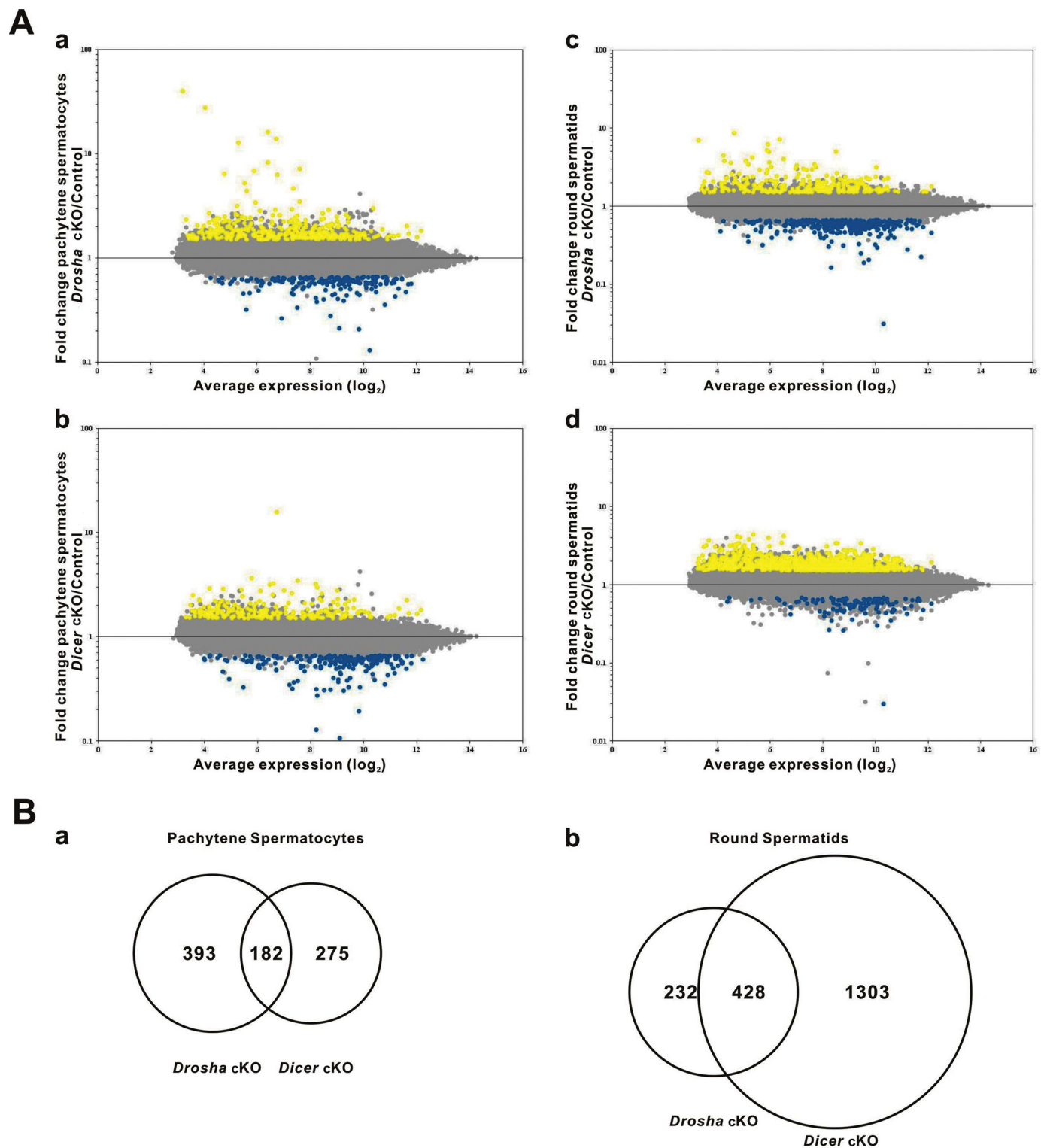
disrupted spermatogenesis accounts for the infertility phenotype of either *Drosha* or *Dicer* cKO male mice.

**Altered mRNA Transcriptomes in *Drosha*- or *Dicer*-null Spermatogenic Cells**—Using the Affymetrix mouse gene 1.0 ST array, we determined the mRNA transcriptomes in pachytene spermatocytes and round spermatids purified from control (heterozygous littermates), *Drosha*, or *Dicer* cKO mice. Among the 28,853 mRNAs probed, 575 and 660 mRNAs displayed significant changes (a fold change  $>1.5$  or less than  $-1.5$ ,  $p < 0.05$  in ANOVA) in *Drosha*-null pachytene spermatocytes and round spermatids, respectively (Fig. 7A, *a* and *c*). 457 and 1731 mRNAs showed significantly altered expression levels in *Dicer*-null pachytene spermatocytes and round spermatids, respectively (Fig. 7A, *panels b* and *d*). There appeared to be more mRNAs with altered expression levels in the *Dicer*-null (a total of 2188) than in *Drosha*-null (a total of 1235) spermatogenic cells (supplemental Table S3). This may suggest that the

absence of both miRNAs and endo-siRNAs in *Dicer*-null spermatogenic cells have a more profound impact on mRNA levels than the lack of miRNAs alone in *Drosha*-null spermatogenic cells.

Despite the fact that some mRNAs showed similar changes in both *Drosha*- and *Dicer*-null spermatogenic cells (Fig. 7B), many mRNAs with altered expression levels appeared to be unique to either of the two genotypes. For example, levels of 393 mRNAs were altered in *Drosha*-null pachytene spermatocytes, and these mRNAs were not changed in *Dicer*-null pachytene spermatocytes. Similarly, 275 mRNAs displayed altered levels in *Dicer*-null pachytene spermatocytes, which were not changed in *Drosha*-null pachytene spermatocytes. Meanwhile, both *Drosha*- and *Dicer*-null pachytene spermatocytes shared 182 mRNAs with significant changes in their expression levels. These differences suggest *Drosha* and *Dicer* may functionally overlap with each other, but they do have their unique impact on steady-state levels of mRNAs, which may reflect the contribution of endo-siRNAs because *Dicer*-null spermatogenic cells lack both canonical miRNAs and endo-siRNAs, whereas *Drosha*-null cells are devoid of canonical miRNAs only. Overall, the microarray analyses revealed that both miRNAs and endo-siRNAs could indeed affect the steady-state levels of mRNAs either directly or indirectly, and this notion is consistent with recent reports demonstrating that miRNAs mainly function to regulate mRNA stability (77). In addition, mRNAs with altered levels in both *Drosha*- and *Dicer*-null cells may reflect the effect of miRNAs, whereas the differences in altered mRNAs may highlight the function of endo-siRNAs. The Affymetrix Mouse Gene 1.0 ST array contains a number of pri-miRNAs, and interestingly, levels of pri-miRNAs for four miRNA clusters, including miR-29 (29a and 29b-1), miR-30 (30b and 30d), miR-34 (34b and 34c), and miR-19 (17, 19b-1 and 92a-1) clusters, displayed 3–10-fold up-regulation in *Drosha*-null pachytene spermatocytes and round spermatids, whereas their levels remained unchanged in *Dicer*-null cells (Fig. 8 and supplemental Table S3). Elevated levels of pri-miRNAs may result from the accumulation of pri-miRNAs due to the lack of DROSHA, which cleaves pri-miRNAs into pre-miRNAs. To validate the microarray data, we chose 43 transcripts identified to be dysregulated in either *Drosha* or *Dicer*-null spermatogenic cells, and we performed qPCR (Fig. 8). Our qPCR results (Fig. 8) were consistent with the microarray data (supplemental Table S3).

**FIGURE 5. Inactivation of *Drosha* or *Dicer* in spermatogenic cells disrupts spermatogenesis.** A, reduced testis size in adult *Drosha* cKO (*Stra8-iCre-Drosha<sup>lox/lox</sup>*) and *Dicer* cKO (*Stra8-iCre-Dicer<sup>lox/lox</sup>*) mice. B, testis weights of P14, P21, and P40 and adult control, *Drosha*, or *Dicer* cKO mice. The number within each column indicates the total number of individual testes measured for each group. C, PAS staining of cross-sections of the *Drosha* cKO testis and cauda epididymis. The control (*Stra8-iCre-Drosha<sup>+/lox</sup>*) testis showed robust spermatogenesis (panel *a*), and the *Drosha* cKO testis displayed severe germ cell depletion (panels *b–d*). Vacuoles (arrows) and multinucleated giant cells (arrowheads), both of which are hallmarks of severe spermatogenic cell depletion, were present throughout the seminiferous epithelium (panels *c* and *d*). Although the control epididymis was filled with spermatozoa (panel *e*), the *Drosha* cKO epididymis contained few spermatozoa and round spermatids probably representing those detached from the seminiferous epithelium and sloughed into the lumen and ended up in the epididymis (panel *f*). Insets show higher magnification of the framed regions in the panels. D, PAS staining of cross-sections of the *Dicer* cKO testis and cauda epididymis. The control (*Stra8-iCre-Dicer<sup>+/lox</sup>*) testis showed normal spermatogenesis (panel *a*), and the *Dicer* cKO testis showed active germ cell depletion (panels *b–d*). Similar to the *Drosha* cKO testis, vacuoles (arrows) and multinucleated giant cells (arrowheads) were noticed in the seminiferous epithelium (panels *c* and *d*). Insets show higher magnification of the framed regions in the panels. The *Dicer* cKO epididymis contained a reduced number of spermatozoa and depleted round spermatids (panel *f*) as compared with the control epididymis (panel *e*). Bars, 20  $\mu$ m. E, percentage of seminiferous tubule cross-sections devoid of elongating or elongated spermatids in control, *Drosha*, and *Dicer* cKO testes. Bars with different letters are statistically significant ( $p < 0.01$ , ANOVA). F, percentage of seminiferous tubule cross-sections containing vacuoles and/or multinucleated giant cells in control, *Drosha*, and *Dicer* cKO testes. Bars with different letters are statistically significant ( $p < 0.01$ , ANOVA).



**FIGURE 7. Altered mRNA transcriptomes in *Drosha*- or *Dicer*-null pachytene spermatocytes and round spermatids.** *A*, microarray analyses revealed numerous mRNAs displaying altered expression levels in *Drosha*-null (*panels a* and *c*) or *Dicer*-null (*panels b* and *d*) pachytene spermatocytes (*panels a* and *b*) and round spermatids (*panels c* and *d*). Each dot represents one mRNA transcript. Yellow dots are those up-regulated mRNAs with a fold change of  $>1.5$  ( $p < 0.05$  in ANOVA), and blue dots indicate the down-regulated ones with a fold change of less than  $-1.5$  ( $p < 0.05$  in ANOVA). *B*, number of mRNAs with significantly altered levels in *Drosha*- and/or *Dicer*-null pachytene spermatocytes (*panel a*) and round spermatids (*panel b*). 575 and 457 mRNAs displayed either up- or down-regulated levels in *Drosha*- and *Dicer*-null pachytene spermatocytes, respectively. Among the 850 mRNAs with altered levels, 182 were found in both *Drosha*- and *Dicer*-null pachytene spermatocytes (*panel a*). Levels of 660 and 1731 mRNAs were altered in *Drosha*- and *Dicer*-null round spermatids, respectively. Among the 1963 mRNAs changed, 428 were shared between the *Drosha*- and *Dicer*-null round spermatids (*panel b*).



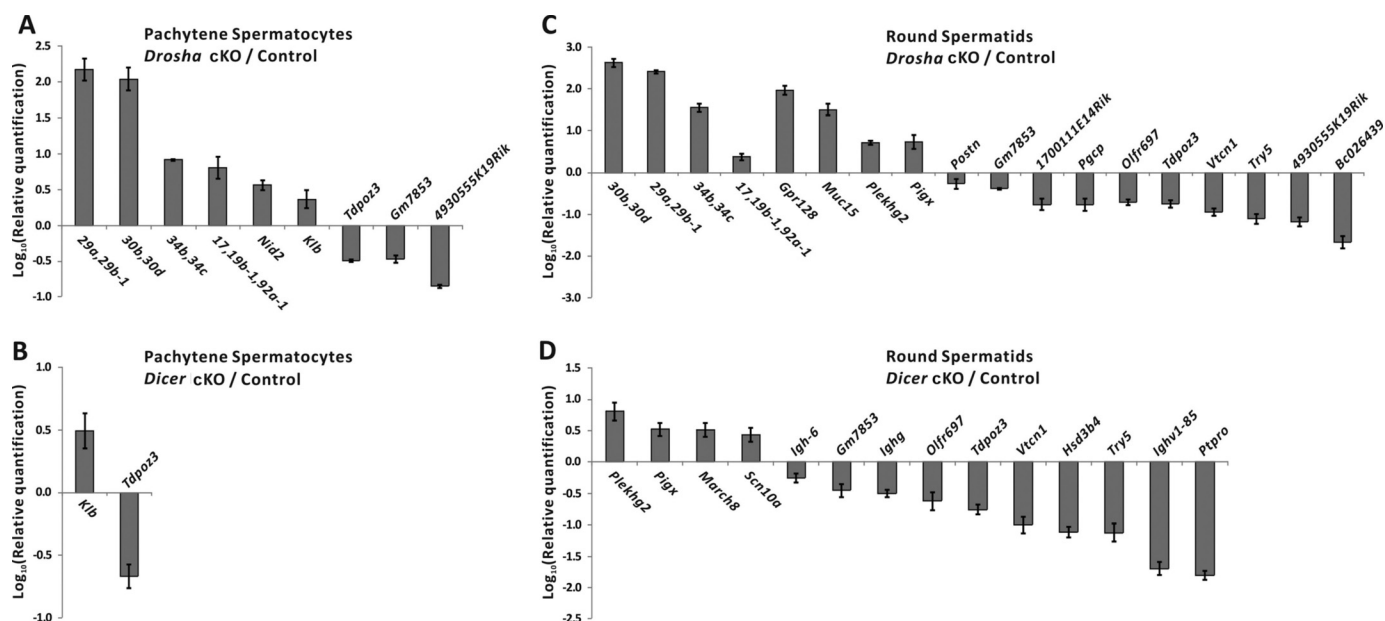


FIGURE 8. Validation of microarray data using SYBR Green-based real time qPCR analyses. A, qPCR validation of altered expression of four pre-miRNAs and five mRNAs in *Drosha* cKO pachytene spermatocytes. B, qPCR validation of altered expression of four pre-miRNAs and 14 mRNAs in *Drosha* cKO round spermatids. C, qPCR validation of altered expression of two mRNAs in *Dicer* cKO pachytene spermatocytes. D, qPCR validation of altered expression of 14 mRNAs in *Dicer* cKO round spermatids. The patterns of changes are consistent with the microarray data.

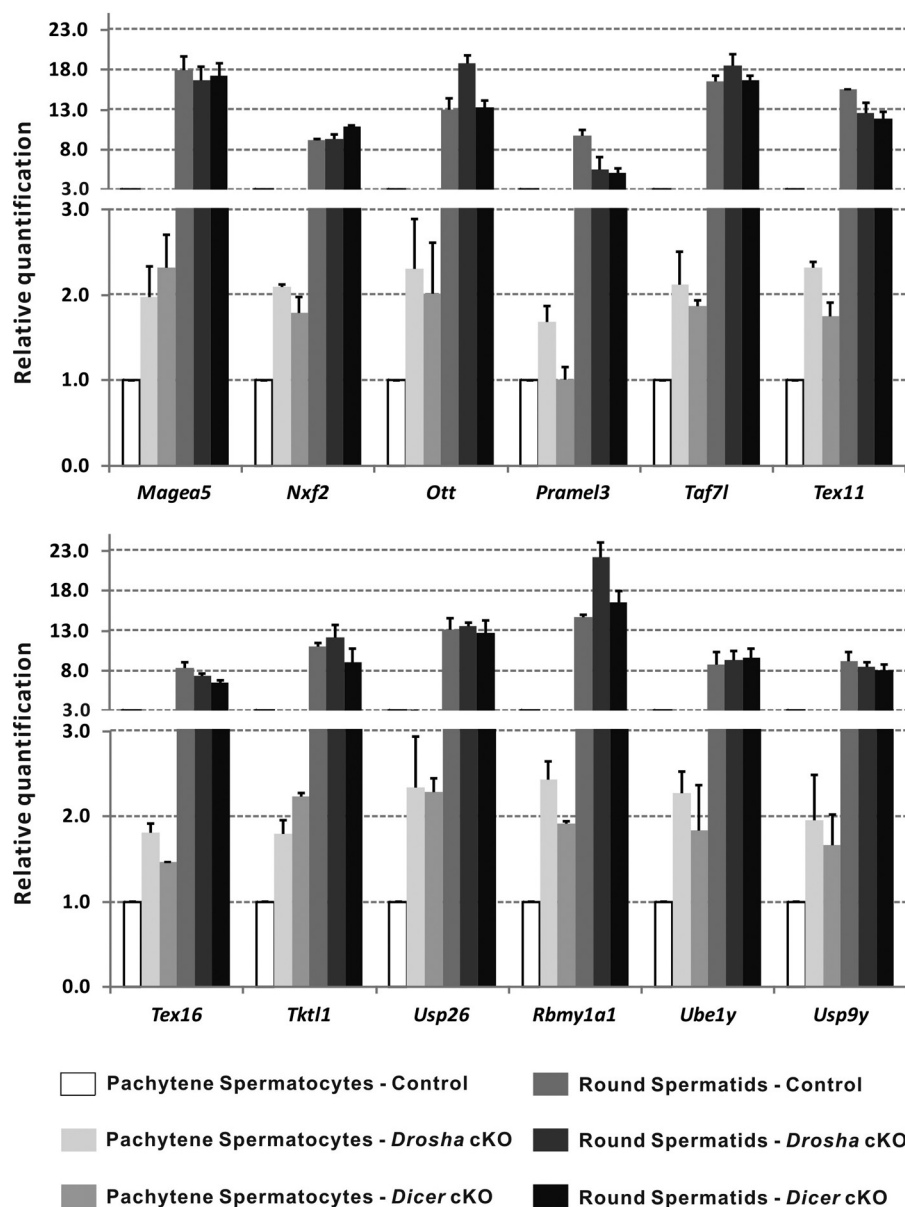
**Sex-linked mRNA Genes Are Up-regulated in Both *Drosha*- and *Dicer*-null Pachytene Spermatocytes**—By examining microarray data, we found several X- or Y-linked genes (e.g. *Ott*, *Nxf2*, *Usp9y*, *Ube1y*, etc.), which are known to be suppressed in pachytene spermatocytes due to meiotic sex chromosome inactivation (MSCI), displayed a 2–4-fold up-regulation in *Drosha*-null pachytene spermatocytes (supplemental Table S3), suggesting a potential defect in MSCI. We therefore chose nine X-linked (*Magea5*, *Nxf2*, *Ott*, *Pramel3*, *Taf7l*, *Tex11*, *Tex16*, *Tktl1*, and *Usp26*) and three Y-linked (*Rbmy1a1*, *Ube1y*, and *Usp9y*) genes and examined their mRNA levels in control, *Drosha*-, or *Dicer*-null pachytene spermatocytes. Levels of these 12 sex-linked mRNAs were indeed up-regulated by ~2-fold in either *Drosha* or *Dicer*-null pachytene spermatocytes (Fig. 9). Both our qPCR data (Fig. 9) and previously published microarray data (78) showed that these 12 mRNAs are drastically up-regulated (by 10–20-fold) in round spermatids following MSCI. The 2-fold up-regulation may not be significant physiologically considering their much higher levels in round spermatids; generally, these sex-linked mRNA genes were still largely suppressed but just to a lesser extent. However, MSCI is known to be essential for meiotic progression, and even a slight relaxation in MSCI may result in disruptions (67, 79). So we examined *de novo* transcription using RNA FISH in conjunction with immunofluorescent staining of  $\gamma$ -H2AX (sex body marker) and HP1 $\beta$  (heterochromatin marker) (67–69, 80). The absence of transcription on the sex chromosomes in mid-pachytene spermatocytes was verified by both Cot-1 (Fig. 10) and *Uty* RNA FISH assays (Fig. 11). Given that the slight up-regulation of sex-linked mRNAs may reflect an MSCI maintenance defect in diplotene spermatocytes, we further examined *de novo* transcription using Cot-1 RNA FISH assays. The sex body remains largely HP1 $\beta$ -positive and Cot-1-negative, suggesting a lack of discernable transcriptional activities even in diplotene stage (Fig. 10). These data suggest that MSCI is established normally in either *Drosha*- or

*Dicer*-null pachytene spermatocytes, and the maintenance of MSCI may have been slightly disrupted, causing the up-regulation of sex-linked mRNAs. But the MSCI defects, if any, may be too minor to be detected due to the limited resolution of the techniques utilized.

**Levels of Transposable Elements Remain Unchanged in Either *Drosha*- or *Dicer*-null Spermatogenic Cells**—Several studies have reported that *Dicer* deficiency in either somatic or germ cells can lead to up-regulated levels of repetitive elements (50, 81, 82). To study whether *Drosha*-deficient spermatogenic cells display transposable element (TE) derepression, we examined levels of IAP, LINE1 (long interspersed nuclear element 1), and SINE B2 (short interspersed nuclear element B2) in purified control, *Drosha*-, or *Dicer*-null pachytene spermatocytes or round spermatids (Fig. 12). Similar to *Dicer*-null pachytene spermatocytes, *Drosha*-null pachytene spermatocytes displayed normal levels of the three types of TEs. Levels of the three types of TEs appeared to be altered in either *Dicer*- or *Drosha*-null round spermatids, but statistical analyses revealed no significant differences (Fig. 12). Consistent with one recent report (49), all three types of TEs showed no significant up-regulation in the testes of a male germ cell-specific *Dicer* cKO mouse line. However, a recent independent study (50) identified the up-regulation of SINE B1 and SINE B2 in *Dicer*-deficient early spermatocytes (*Ddx4-Cre-Dicer<sup>lox/lox</sup>*) and up-regulation of SINE B1, SINE B2, and LINE1 in *Dicer*-deficient late spermatocytes. The discrepancy may result from the onset of *Dicer* inactivation due to different Cre lines used.

## DISCUSSION

**Lighting up Knock-out (KO) Cells with Fluorescent Proteins Allows Monitoring Cellular Changes in Vivo and Purifying KO Cells for Molecular Analyses**—The Cre-loxp-mediated cKO system allows the inactivation of a gene of interest along a spe-



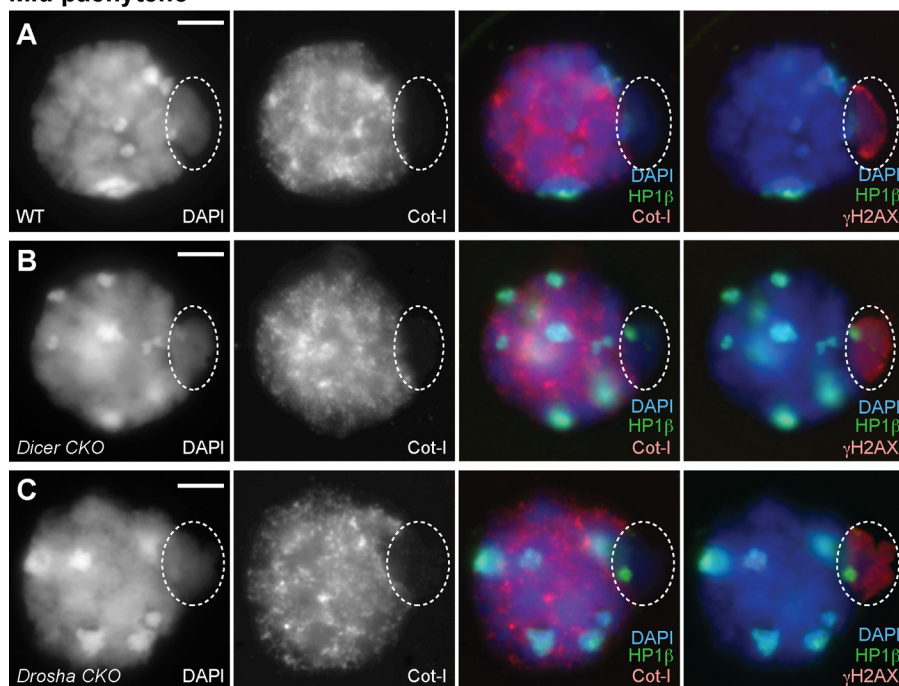
**FIGURE 9. qPCR analyses of expression levels of nine X- and three Y-linked mRNA-coding genes in control, *Drosha*-, or *Dicer*-null pachytene spermatocytes and round spermatids.** All of the nine X-linked genes (*Magea5*, *Nxf2*, *Ott*, *Pramel3*, *Taf7l*, *Tex11*, *Tex16*, *Tktl1*, and *Usp26*) and three Y-linked genes (*Rbmy1a1*, *Ube1y*, and *Usp9y*) examined have been shown to be largely suppressed during MSCI, and thus their levels in pachytene spermatocytes are at minimal levels. But once MSCI is completed, these genes are highly expressed in round spermatids. Levels of these genes in control pachytene spermatocytes were used as the normalizer. *Gapdh* was used as an internal control. The experiments were performed in biological triplicates.

cific cell lineage and thus can overcome the embryonic lethality problem often associated with the global/universal knock-out approach. Although humans with a null mutation in *DGCR8* are viable, both *Dicer* and *Dgcr8* global inactivation in mice leads to embryonic lethality (24, 46), thus precluding the analyses of their functions during germ cell development. The routine method of determining Cre activity is to cross a Cre line with a reporter line that expresses either an enzyme (e.g.  $\beta$ -galactosidase, luciferase, etc.) or a fluorescent protein (e.g. EGFP, tomato Red, etc.), and reporter-expressing cells are automatically assumed to represent the site of Cre activity in the cKO mice. Several cKO mouse lines in which *Dicer* was selectively inactivated in the male germ line during fetal or postnatal development have been generated (49–53). However, one of

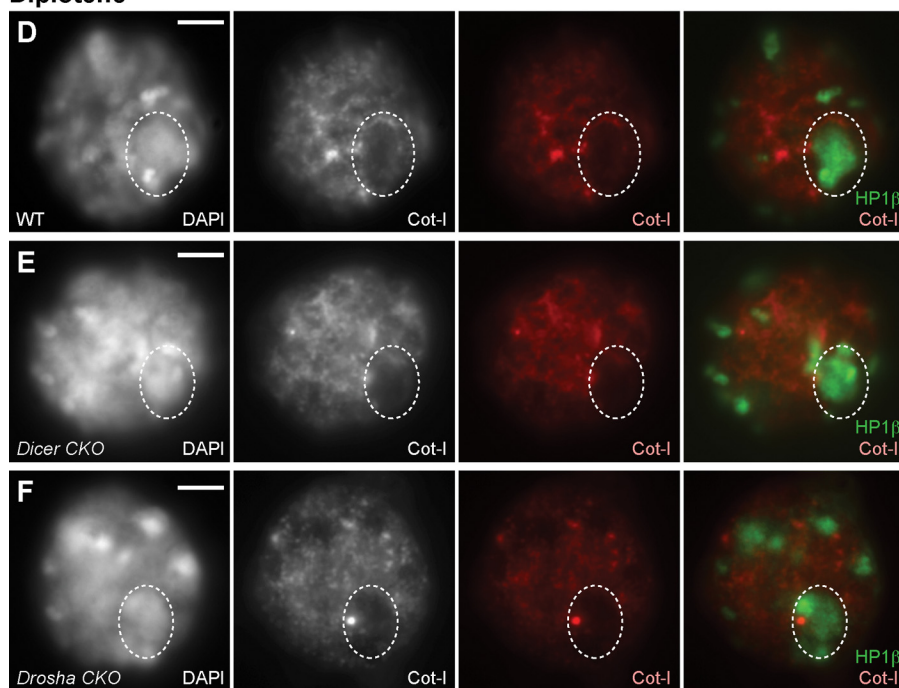
the Cre mouse lines used is known to display partial penetrance of Cre expression and/or Cre activity in the targeted cell lineage (83). The incomplete Cre penetrance can lead to mosaicism/hypomorphism, and the phenotype observed thus cannot reflect the true effects of a complete ablation of *Dicer*. In addition, three of these studies used total testes to conduct the molecular analyses (49, 52, 53). Given that the total testes contain both somatic and developing male germ cell types, and the true cKO cells are only a proportion of the total testicular cells, analyses performed using total testes rendered the true effects being masked by normal or changed expression in other non-cKO cell types. Thus, phenotypes observed and molecular analyses performed more likely represent a hypomorphic scenario. In this study, by introducing the dual fluorescence reporter



## Mid pachytene



## Diplotene



**FIGURE 10. Evaluation of MSCI in control (WT), *Drosha*-, or *Dicer*-null mid-pachytene and diplotene spermatocytes using double immunofluorescence with anti- $\gamma$ H2AX and HP1 $\beta$  antibodies followed by Cot-1 RNA FISH.** A–C, in mid-pachytene spermatocytes, XY body becomes prominent and protrudes from the rest of the nuclei in the mid-pachytene stage. Cot-1 signal was almost completely depleted from the XY body, and  $\gamma$ H2AX accumulated on the XY body, and HP1 $\beta$  only localized on X-centromere at this stage (small dots in the XY body). No significant difference was observed among controls (A), *Drosha*-null (B), and *Dicer*-null (C) mid-pachytene spermatocytes, suggesting MSCI was initiated and developed normally in either *Drosha*- or *Dicer*-null pachytene spermatocytes. D–F, in the diplotene phase, HP1 $\beta$  was localized to the entire XY body. XY body became DAPI-intense and moved toward inside of the nuclei. Depletion of Cot-1 signals was the same between control and mutants. More than 50 cells with HP1 $\beta$  negativity on the XY body (mid-pachytene) and >50 cells with HP1 $\beta$  positivity on XY body (late pachytene to diplotene) were examined. However, no clear difference was detected between control and mutants. Dotted circle, XY body. Bars, 10  $\mu$ m. >50 nuclei were counted, and all displayed the same staining patterns as those presented in this figure.

allele (mT/mG) into the loxp homozygous background, we generated two compound cKO lines, in which Cre-expressing cells expressed membrane-bound EGFP (mG) and Cre-negative cells expressed membrane-bound tomato red (mT) fluores-

cence. In this way, we could not only monitor the developmental course of cellular disruptions *in vivo* (Fig. 1) but also purify the cKO cells based upon mG or judge purity after purification based upon the ratio of green *versus* red cells (Fig. 2). It is note-

## Male Germ Line Ablation of *Drosha* Impairs Spermatogenesis

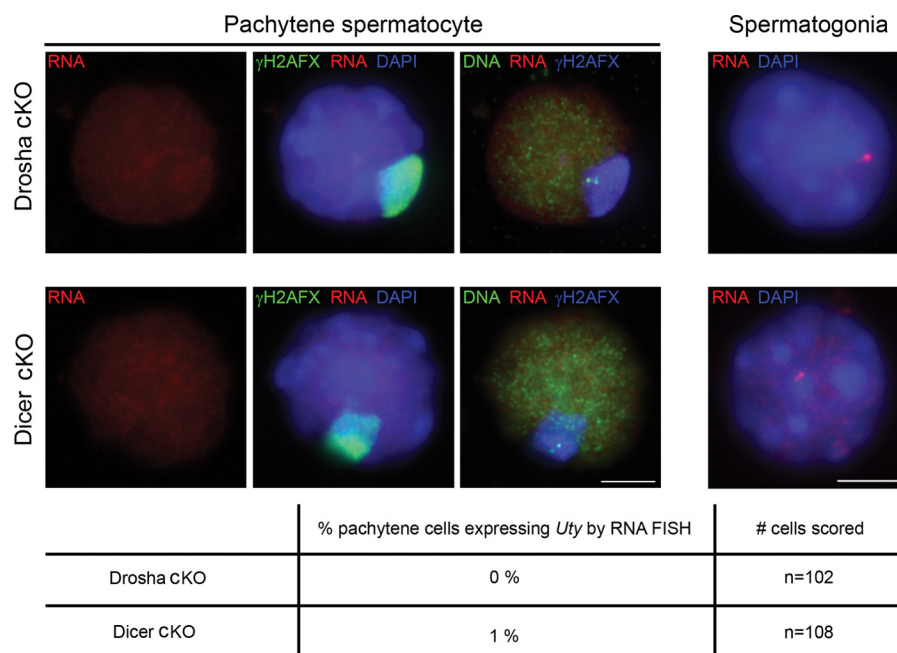


FIGURE 11. RNA FISH analyses on *Drosha*- or *Dicer*-null pachytene spermatocytes and spermatogonia (control). Ongoing transcription of the Y-linked gene *Uty* was detected in spermatogonia but not in either *Drosha*- or *Dicer*-null pachytene spermatocytes. The sex body showed normal  $\gamma$ H2AFX immunoreactivity (green). Bars, 5  $\mu$ m.

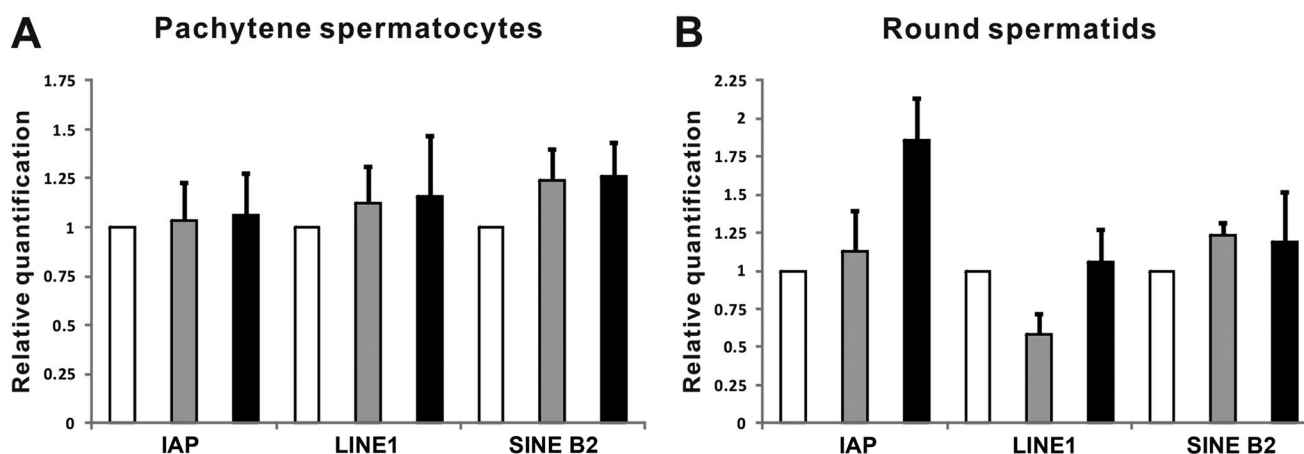


FIGURE 12. Levels of transposable elements in *Drosha*- or *Dicer*-null pachytene spermatocytes and round spermatids. A, qPCR analyses of IAP, LINE1, and SINE B2 in control, *Drosha*-, or *Dicer*-null pachytene spermatocytes. B, qPCR analyses of IAP, LINE1, and SINE B2 in control, *Drosha*-, or *Dicer*-null round spermatids.

worthy that mG was initially expressed only in a small proportion of spermatogonia at P4, and its expression gradually occurred in most of spermatogonia during early postnatal development. In adult testes, levels of mG were much lower in spermatogonia than in spermatocytes and round spermatids (Fig. 1). Collectively, these data suggest that inactivation of *Drosha* or *Dicer* in spermatogonia using the *Stra8*-Cre line as a deleter is likely incomplete, and thus spermatogonia may have remained partially functional, which was sufficient to support their development. Therefore, the lack of discernable morpho-

logical disruptions in spermatogonial populations cannot exclude a role for *Drosha* or *Dicer* in the mitotic phase of spermatogenesis. In contrast, the full penetrance of Cre activity in spermatocytes and spermatids was consistent with the fact that these two cell types were those showing the most severe depletion in developing and adult cKO testes. In addition, the highly pure (>90% of purity) pachytene spermatocytes and round spermatids allowed us to determine expression levels of small RNAs and sex-linked mRNAs, as well as microarray analyses of the whole mRNA transcriptome in the cKO cell types rather



than in the total testes. Generation of cKO lines with reporter capability represents an improved way of studying the effects of cell- or tissue-specific inactivation of a particular gene because it allows *in vivo* observation of the time course and the degree of cellular disruptions and easy purification of the cKO cells for molecular analyses.

**Phenotypic Differences between *Drosha* and *Dicer* cKO Germ Cells May Reflect the Role of Endo-siRNAs**—By morphological and transcriptomic analyses, we identified differences between *Drosha* and *Dicer* cKO testes (Figs. 5–7). The differences may reflect the effects of deficiency in endo-siRNAs because *Drosha*-null spermatocytes and round spermatids only lack canonical miRNAs, whereas *Dicer*-null spermatocytes and spermatids are devoid of not only of canonical miRNAs but also endo-siRNAs and maybe other DICER-dependent sncRNAs. A comparative study of the phenotype of *Dicer* and *Dgcr8* cKO females has revealed that although inactivation of *Dicer* in developing oocytes leads to female infertility due to oocyte development/maturation defects, *Dgcr8*-null oocytes develop normally and can be fertilized by WT sperm and produce normal pups (46, 82). Two subsequent studies have demonstrated that miRNA functions are largely suppressed in developing oocytes and even in preimplantation embryos, whereas endo-siRNAs are required for the same processes (47, 48). Given the fact that *Drosha* cKO testes displayed more severe morphological disruptions (Figs. 5 and 6), it is plausible to speculate that the male germ cell-expressed endo-siRNAs may have a “protective” effect because the lack of miRNAs alone in *Drosha* cKO mice appeared to cause more severe disruptions to spermatocytes and round spermatids than the ablation of both miRNAs and endo-siRNAs in *Dicer* cKO testes. Nevertheless, the time course and severity of spermatogenic disruptions in these two cKO lines were largely similar, which is consistent with the fact that these two RNase III enzymes are involved in a common pathway, *i.e.* canonical miRNA biogenesis (73). Supporting this notion, mRNA transcriptomic analyses revealed that many of the altered mRNAs are shared between cKO spermatocytes (182 mRNAs) and round spermatids (428 mRNAs) (Fig. 7 and supplemental Table S3) of the two genotypes. However, the *Drosha*-null pachytene spermatocytes and round spermatids indeed have their unique transcriptomes represented by 393 mRNAs in pachytene spermatocytes and 232 mRNAs in round spermatids that were not changed in *Dicer*-null pachytene spermatocytes and round spermatids, respectively. It is noteworthy that *Dicer*-null round spermatids had 1303 altered mRNAs, levels of which were not changed in *Drosha*-null round spermatids, suggesting the absence of both canonical miRNAs and endo-siRNAs can cause more changes in mRNA levels than the lack of canonical miRNAs alone (1731 *versus* 660 mRNAs). In general, the effects of *Drosha* deficiency on mRNA levels in both pachytene spermatocytes and round spermatids were the same (575 *versus* 660 mRNAs). In contrast, the *Dicer* absence displayed much more profound impact on the mRNA levels in round spermatids than in pachytene spermatocytes (1731 *versus* 457 mRNAs). These data indicate that miRNAs and/or endo-siRNAs affect, either directly or indirectly, the steady-state levels of mRNAs mainly in round spermatids in the adult testes.

**Like Endo-siRNAs, miRNAs Can Affect Steady-state Levels of mRNAs**—Previously, it was believed that miRNAs mainly function as post-transcriptional regulators by binding to the 3'UTRs and induce translational suppression (17, 84). Recent data, however, have demonstrated that some miRNAs can increase translation efficiency (85), and miRNAs generally can enhance the stability of their target mRNAs (77). Endo-siRNAs theoretically can induce mRNA degradation through the RNAi mechanism (54, 86, 87). The fact that the *Drosha* deficiency alone (lack of canonical miRNAs only) can induce changes in ~600 mRNAs in either pachytene spermatocytes (575 mRNAs) or round spermatids (660 mRNAs) suggests that miRNAs have a role in the mRNA stability control. More mRNAs were altered in *Drosha*-null pachytene spermatocytes than in *Dicer*-null pachytene spermatocytes (575 *versus* 457 mRNAs), which is consistent with the more severe morphological disruptions in *Drosha* than in the *Dicer* cKO testes observed (Fig. 5). In contrast, *Dicer* inactivation, which led to deficiency in both miRNAs and endo-siRNAs, caused changes in ~1700 mRNAs in round spermatids, which is much more than that in *Drosha*-deficient round spermatids (660 mRNAs), suggesting that endo-siRNAs can cause more changes in mRNA levels. This is consistent with the fact that endo-siRNAs are directly associated with mRNA stability. Also, this implies a more significant role of endo-siRNAs in round spermatids than in pachytene spermatocytes in the regulation of mRNA stability. Among these mRNAs with altered levels, the majorities displayed up-regulation (Fig. 7), suggesting that endo-siRNAs and/or miRNAs can induce mRNA degradation.

**miRNAs and/or Endo-siRNAs Are Not Essential for the Initiation of MSCI but May Be Involved the Maintenance of MSCI**—An ~2-fold increase in mRNA levels of many X- or Y-linked genes in both *Drosha*- and *Dicer*-null pachytene spermatocytes suggests a relaxation of the suppressive status due to MSCI. However, all the genes examined display de-suppression once the male germ cell development progresses from spermatocytes to round spermatids, which have been documented in a previous transcriptomic study (78) and this study (Fig. 9). In round spermatids, the levels of these X- or Y-linked genes are ~8–16-fold higher than their levels in pachytene spermatocytes. Therefore, when these much higher levels were compared with much lower levels in pachytene spermatocytes, an ~2-fold increase is still 4–8-fold lower than levels in round spermatids, indicating that these genes were still largely suppressed, and the disruption in MSCI is thus minimal, as reflected by the mild increase in X- or Y-linked mRNA gene expression (Fig. 9). Consistent with this notion, analyses on MSCI using two independent RNA FISH techniques (Figs. 10 and 11) demonstrated a lack of *de novo* transcription and normal formation of the sex body in either *Drosha*- or *Dicer*-null pachytene spermatocytes. These data suggest that miRNAs and/or endo-siRNAs are not required for the establishment of MSCI. But in the absence of these sncRNAs, the maintenance of MSCI may have been compromised to some extent. However, these potential disruptions appeared to be subtle/minor, which are beyond the detection sensitivity/resolution of the techniques used (*i.e.* RNA-FISH in conjunction with immunofluorescent staining). The potential minor MSCI defects may con-

tribute to the severe depletion of pachytene spermatocytes observed in both *Droscha* and *Dicer* cKO testes because defective MSCI is not compatible with meiotic progression and thus MSCI-defective spermatocytes must be eliminated through apoptosis by the meiotic checkpoint mechanism (67, 79). The fact that many spermatocytes managed to form round spermatids suggests that the disruption of MSCI is mild.

In summary, data from this study demonstrate an essential role of *Droscha* in canonical miRNA biogenesis *in vivo*, and the deficiency in *Droscha* or the lack of DROSHA-dependent miRNAs can lead to disrupted spermatogenesis and male infertility. Like *Dicer*, *Droscha* is essential for both the meiotic and haploid phases of spermatogenesis. Endo-siRNAs appear to have more profound impact on the mRNA stability than miRNAs do in round spermatids, suggesting a critical role of this novel sncRNA species in the regulation of mRNA stability in the haploid phase of spermatogenesis. The phenotypes observed may represent direct or indirect effects of canonical miRNA and/or endo-siRNAs deficiency. Nevertheless, our data, together with other similar studies (49, 50), demand further investigations on the molecular mechanism underlying the spermatogenic defects observed in both *Droscha*- and *Dicer*-deficient testes.

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