Editorial

Editorial on "PNLDC1 is essential for piRNA 3' end trimming and transposon silencing during spermatogenesis in mice"

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Piwi-interacting RNA (piRNA) are a class of small non-coding RNAs expressed in the germline. These piRNAs are processed to a mature piRNA by endonucleases and exonucleases that are bound to PIWI family proteins (1-3). In mouse, three Piwi proteins have been shown to be critical for spermatogenesis, namely Miwi, Mili and Miwi2 (4-6). Mili and Miwi show different temporal expression during testes development. Mili is expressed earlier until the pachytene stage of meiosis, and Miwi is expressed from pachytene until the haploid round spermatid stage. Disruption of the genes encoding Mili and Miwi results in sterile males due to spermatogenesis arresting at the pachytene stage, resulting from a failure of piRNA retrotransposon silencing (5-11). Transposon silencing within the germline requires the correct processing of piRNA. Immature piRNA requires 3'-5' trimming to generate mature piRNAs (12-14). A protein related to the poly(A)-specific ribonuclease (PARN) family, poly(A)-specific ribonuclease-like domain containing 1 (PNLDC1), has been recognized as an enzyme "trimmer" for pre-piRNA 3' end trimming and maturation processing in silkworms (14). PNLDC1 has also been found to be expressed in mammalian species including mouse and human (15). The potential role of PNLDC1 in mammals was unclear until recent reports have implicated it in piRNA processing in mice during spermatogenesis (16-18).

The expression pattern of *Pnldc1* has been specifically found in the testis tissue of mice (18). Furthermore, the expression is higher in spermatogonial stem cells compared to that in pachytene spermatocytes and round spermatids (18). Ding *et al.* utilized CRISPR-Cas9 genome editing to generate *Pnldc1*-deficient mice in order to investigate the potential role of PNLDC1 in piRNA processing and subsequent spermatogenesis (16). Five male $Pnldc1^{Mut}$ mice were examined that all had distinct mutations on *Pnldc1* alleles. All were viable and grew normally but had 30% smaller testes compared to the control mice by weight. $Pnldc1^{Mut}$ mice also lacked normal spermatozoa and germ cell numbers were drastically reduced (16). Male mice that were *Pnldc* knockout mice also displayed similar spermiogenic arrest as was observed in the $Pnldc1^{Mut}$ males. Independently also using CRISPR-Cas9 Nishimura *et al.* produced *Pnldc1* mutant mice. Adult $Pnldc1^{Mut}$ males had testis that were approximately half the size of age matched wildtype mice (17). Similarly they found $Pnldc1^{Mut}$ mice were not able to produce mature sperm. Finally, a third study also found *Pnldc1* knockout mice were unable to produce mature sperm and had significantly smaller testes (18). In all case defects were seen in both meiosis and spermiogenesis, supporting that *Pnldc1* plays an important role in these processes.

Long interspersed nuclear element 1 (LINE1) is the dominant transposable element in the DNA of placental mammals including mice. Ding *et al.* utilized LINE1 messenger RNA (mRNA) expression to determine the ability of the piRNA pathway to silence transposons. LINE1 was measured in adult $Pnldc1^{Mut}$ testes to examine possible roles of *Pnldc1* in transposon silencing. Significant elevation in LINE1 mRNA expression was observed in $Pnldc1^{Mut}$ spermatocytes. Western blot results also revealed increased LINE1 protein levels in $Pnldc1^{Mut}$ testes. This is consistent with the findings of that LINE1 protein was increased at postnatal day 14 in $Pnldc^{Mut}$ mouse testes (17). Similarly, Zhang *et al.* found a significant increase in LINE1 mRNA and protein in *Pnldc* knockout mice testes from postnatal day 18 increasing to day 60 (18). These results indicate, in mammals, that PNLDC1 is required to correctly repress

transposons in male germ cells.

The size and quantity of the small RNA populations in adult wild-type and *Pnldc1*^{Mut} testes were compared to explore the role of PNLDC1 in piRNA maturation. In wild-type mice the piRNA population found to be ~30 nt in length. Interestingly, in *Pnldc1*^{Mut} testes a longer small RNA population of 30–40 nt was observed. Further support is provided by Nishimura *et al.* who utilized deep sequencing to analyze the size distribution of small RNAs (17). They found the majority of small RNAs were 24–31 nt in control mice whereas they were distributed from 24–50 nt in the mutant embryonic testes (17). Furthermore, these findings were independently verified in a study by Zhang *et al.* who found a reduction in the population of 24–32 nt small RNAs and an increase in the small RNAs >30 nt, in the Pnldc1 knockout mice (18). This indicated a role for PNLDC1 in the production of mature piRNAs and the facilitation of pre-piRNA processing.

Genome mapping of the small RNA libraries for wild-type and *Pnldc1*^{Mut} mice was done to examine the extended piRNA species. The first nucleotide composition was measured specifically in the *Pnldc1*^{Mut} pre-piRNAs, and piRNAs from both 24–32 and 33–40 nt reads had a U bias. This suggested the 3' end was incorrectly processed in the *Pnldc1*^{Mut} species. To test this, 5' end match analysis was performed on piRNA from wild-type testes and *Pnldc1*^{Mut} testes. It was found that while the 5' ends had similar nucleotides in the wild-type and *Pnldc1*^{Mut} piRNAs, the 3' end was variable. This strongly supports a role for PNLDC1 in pre-piRNA 3' end trimming. It is important to note that two other studies arrived at the same conclusion, namely that PNLDC1 is required for the correct 3' processing of piRNAs (17,18).

To explore the pachytene piRNA precursor cleavage mechanism, untrimmed pachytene pre-piRNAs in *Pnldc1*^{Mut} mice were analyzed by genomic mapping (16). Wild-type and *Pnldc1*^{Mut} small RNA libraries were mapped using 24–48 nt reads. They were also analyzed at the 5' and 3' end sequences from mapped piRNAs. It was found that U residues are enriched in the first position downstream of the 3' end in the *Pnldc1*^{Mut} piRNA. This is a signature of phased piRNA processing and is known to be linked to the production of phased pre-pachytene piRNAs. This phased pachytene piRNA biogenesis was confirmed by further coupling analysis. The piRNA 5' ends in wild-type mice were distributed randomly around the upstream piRNA 3' ends, however, in untrimmed *Pnldc1*^{Mut} pre-piRNAs, the 5' ends were enriched immediately downstream of the piRNA 3' ends. These results suggest a single cleavage event generates the 3' end of one piRNA and the 5' end of the next downstream piRNA. This supports phased biogenesis of piRNA contributing to mammalian pachytene piRNA production.

Neonatal *Pnldc1* heterozygous and *Pnldc1* homozygous knockout mutant mice were analyzed to investigate PNLDC1's role in pre-pachytene piRNA production. Both MIWI and MILI mislocalization was observed in knockout mice with expression in large perinuclear granules consisting of clustered mitochondria (16). However, while the expression level of Mili was similar in the control and mutant testes, the expression of Miwi was significantly decreased in the mutant testes (16). As expected Mili bound *Pnldc1* knockout piRNAs had an extended nucleotide length compared to the control, indicating a piRNA trimming defect. Mapping of piRNAs from *Pnldc1* knockout testes found 3' end extensions supporting a deficiency in 3' end trimming. LINE1 immunostaining was performed on *Pnldc1* knockout germ cells, and no upregulation was observed. These results suggest that PNLDC1-deficient neonatal male germ cells have functional LINE1 silencing but defective piRNA trimming along with incomplete MIWI2 localization. In all three studies it appears that Miwi is more sensitive to PNLDC1 loss when compared to Mili (16-18). Due to Mili being expressed prior to Miwi it is possible that it facilitates piRNA processing for interaction with Miwi and the loss of PNLDC1 destabilizes this process.

The intercellular activities of PNLDC1 remain largely undefined. It has been proposed to play a role in the maintenance of multipotency, genome surveillance, and re-programming in early development (15). Recently, PNLDC1 was reported to provide the elusive 3' pre-piRNA trimming activities in *B. mori*, *C. elegans*, and silkworm (14,15). In recent reports using PNLDC1 mutant mice, three independent studies have confirmed a role in piRNA processing for PNLDC1 (16-18). It has shown to be critical for the correct meiosis and spermiogenesis in mice. It was demonstrated that PNLDC1 is required for the 3' end maturation of piRNAs. This results in a failure to silence the LINE1 transposon and male sterility. It is important to note that female *Pnldc1* mutants display apparent normal fertility. These recent results provide new insights into the critical functions of piRNA in both meiosis and spermatogenesis.

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Footnote

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