Editorial

Crosslinking and immunoprecipitation: a new route for dead end

Di Chen, Grace Hancock

Department of Molecular Cell and Developmental Biology, University of California, Los Angeles, USA

Correspondence to: Di Chen. Department of Molecular Cell and Developmental Biology, University of California, Los Angeles, USA.
Email: deechen@ucla.edu.

Comment on: Yamaji M, Jishage M, Meyer C, *et al.* DND1 maintains germline stem cells via recruitment of the CCR4-NOT complex to target mRNAs. Nature 2017;543:568-72.

Received: 15 June 2017; Accepted: 04 August 2017; Published: 24 August 2017..
doi: 10.21037/amj.2017.08.07
View this article at: http://dx.doi.org/10.21037/amj.2017.08.07

Germline cells are the foundation of inheritance for genetic and epigenetic information from generation to generation. The precursors of germ cells, termed primordial germ cells (PGCs), are one of the earliest cell types specified during embryogenesis. There are two modes of PGC origin. In model organisms such as Drosophila, C. elegans, zebrafish, and Xenopus, germ cells are specified by the inheritance of germ plasm, which is composed of maternal proteins and RNAs. In contrast, in mouse and possibly most mammals including humans, PGCs are induced by local signals such as BMP4 and WNT (1). After specification by either strategy, PGCs proliferate to increase in number, migrate to form gonads with somatic cells, undergo meiosis to halve their genetic material (DNA), and differentiate to mature gametes. At the molecular level, PGCs undergo dramatic epigome reprogramming including genome-wide DNA demethylation and histone modification rewiring (2,3). Any mistake that happens during the abovementioned steps could ultimately lead to sterility due to germ cell loss or germ cell tumors.

RNA-binding proteins (RBPs) are abundantly expressed in germ cells across species. RBPs bind mRNAs to regulate their localization, stability, and translation efficiency at different steps of development. Some of these RBPs are specifically expressed in germ cells and are routinely used as germ cell markers, such as VASA, PIWI family members, NANOS family members, and dead end homolog 1 (DND1) (4). However, understanding the molecular function of these RBPs in germline development is rendered by the difficulty of characterizing their *in vivo* functional targets. Crosslinking and immunoprecipitation (CLIP) of RNAs bound by the RBPs is the standard methodology to identify target RNAs (5). However, CLIP technique requires large cell number and high-quality antibodies for RBPs. Due to these two limitations, it is difficult to perform CLIP on mammalian early germ cells, especially PGCs, to identify the RNA targets. Recently, Yamaji *et al.* published a paper that applied modified CLIP to identify the RNA targets of DND1 in human and mouse culture cells (6), providing new insights to the function of RBPs in germline regulation.

In mice, DND1 is expressed from the nascent stage in PGCs until the pre-meiotic spermatogonia stage in testis. DND1 mutant mice have deficient germlines, with a PGC loss phenotype in all genetic backgrounds, and growth of testicular germ cell tumors in some genetic backgrounds (6-8). This expression pattern and mutant phenotype suggests that DND1 is critical for mouse PGC maintenance and development. Similarly, DND1 mutation in rats also leads to germ cell tumors and germline failure (9). The molecular function of mammalian DND1 was largely unknown however, until the identification of DND1-binding RNAs, and interacting proteins as discovered by Yamaji *et al.* 2017 (6). Additionally, zebrafish DND1 is specifically expressed in PGCs, and knockdown of DND1 exhibits a germline failure phenotype (10), suggesting that DND1 plays a conserved role in regulating germ cells in vertebrates. In zebrafish, DND1 binds 3'UTR of certain mRNAs to protect these mRNAs from miRNA-mediated repression (11). This regulation is also observed in human cells (11).

Tut is the putative Drosophila homolog of DND1, although the conservation to higher organisms is low (12). Interestingly, Tut is specifically expressed in spermatogonia

and male Tut mutants are sterile with severe germ cell tumors, highly resembling the testicular germ cell tumor phenotype observed in *DND1* mutant mice. Tut interacts physically with two other RBPs, Bam and Bgcn, to form an RBP complex that regulates the proliferation and differentiation of germline cells, possibly acting through RNAs. One of the RNA targets of the Tut-Bam-Bgcn complex is the *mei-P26* mRNA. *mei-P26* is another RBP and *mei-P26* mutants are also male sterile with germ cell tumors. Interestingly, in Tut mutant testis, *mei-P26* mRNAs have longer 3'UTR compared to that of wild-type, suggesting that Tut may regulate the alternative polyadenylation in germ cells (12). Moreover, the Tut-Bam-Bgcn complex recruits DDR4-NOT deadenylase machinery to destabilize target mRNAs in germline cells (13).

Studies in mouse, zebrafish, and Drosophila indicate the importance of DND1 in germline development through regulating mRNA. However, understanding the molecular function of DND1 in germline development requires transcriptome-wide identification of DND1-binding targets in germ cells. A recent study applied photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) to characterize DND1-binding targets in both human and mouse (6). They first made a stable human cell line HEK293 with inducible expression of human DND1 tagged with Flag and hemagglutinin (HA). By controlling the expression level of DND1 in this cell line, the authors performed PAR-CLIP and identified DND1-binding RNAs. About 90% of the DND1-binding RNAs contain a UU (U/A) binding motif. In order to test how DND1 regulates mRNA expression, the authors performed RNA-sequencing (RNA-seq) and found that the DND1-binding mRNAs were down-regulated when DND1 was expressed, suggesting that DND1 destabilizes target mRNAs. Only the motif UU (U/A) in the 3'UTR region contributed to this DND1-mediated down-regulation, and mRNAs with more UU (U/A) sites were also more down-regulated. To understand how DND1 destabilizes target mRNAs, the authors characterized DND1 interacting partners by mass spectrometry. Interestingly, DND1 interacts with the CCR4-NOT complex, consistent with the finding in Drosophila (13). One of the main functions of the CCR4-NOT complex is to destabilize mRNA through deadenylation (14). Importantly, knocking-down of CCR4-NOT complex components abrogated DND1-mediated repression (6) , suggesting that DND1 destabilizes target mRNAs through the CCR4-NOT complex. The authors also did similar analyses of mouse DND1 in cultured mouse cells and found that DND1-binding mRNAs and DND1 molecular function is conserved in mammals.

Based on functional characterization of DND1 in *in vitro* cultured somatic cells, the authors analyzed the function of mouse DND1 in spermatogonial stem cells. They characterized the transcriptomes of more than 100 individual spermatogonia by Drop-seq and found that DND1 expression level was inversely correlated with DND1-target mRNA expression, further confirming the destabilization function of DND1. Additionally, knocking-down of DND1 leads to apoptosis and loss of spermatogonial stem cells. Interestingly, DND1 targets include positive regulators of apoptosis. Importantly, knocking-down CCR4-NOT components leads to a similar mRNA profile change as knocking-down DND1, supporting the model that DND1 recruits CCR4-NOT complex to destabilize target mRNAs in maintaining spermatogonia. This anti-parallel expression dynamic between DND1 and DND1-binding mRNAs was also observed in PGC-like cells (PGCLCs) induced from embryonic stem cells (ESCs) through epiblast-like cells. When inducing PGCLCs from DND1 mutant ESCs, the PGCLCs expressed PGC markers such as Prdm1, Prdm14 and Nanos3, suggesting that germ cell fate induction was not affected. However, the expression of DND1 target mRNAs that were down-regulated during wild-type PGCLC development was elevated in DND1 mutant PGCLCs. These observations suggest that the analysis of DND1 function in cultured somatic cells can be applied to germline cells.

In summary, Yamaji *et al.* 2017 discovered that DND1 functions with the CCR4-NOT complex to destabilize mRNAs. The next important questions will be what the DND-binding targets are and why they need to be destabilized in germline cells. Yamaji *et al.* found that the top 300 DND1 targets are associated with signaling pathways regulating pluripotency and cancer development, such as TGF and WNT signaling. Elimination of these mRNAs may be related to the cell fate transition from the pluripotent to PGC state. DND1-binding targets also include positive regulators of apoptosis. DND1-mediated destabilization of these pro-apoptotic factors may be important for the survival of germ cells. Overall, the authors discovered DND1 recruits CCR4-NOT complex to mRNAs with UU (U/A) motifs on 3'UTR for destabilization in cultured cells, and found the same functional relationship of DND1 and DND1-binding

mRNAs in germline cells. Because all of the analyses in germ cells are based on the DND1-binding mRNAs identified from *in vitro* cultured somatic cells, it will be important to characterize DND1-binding mRNAs in germ cells by modifying PAR-CLIP technique for small cell numbers. This will also distinguish DND1-binding mRNAs in different stages of germ cell development, such as PGCs and spermatogonial stem cells. The homolog of DND1 in zebrafish has been found to bind mRNA 3'UTR and release it from miRNA-mediated repression (11), thus functioning in a positive regulatory way. It would be interesting to analyze the DND1-binding mRNAs that are up-regulated when DND1 are expressed in *in vitro* cultured cells. These mRNAs may be protected from miRNA-mediated repression by DND1. Since DND1 functions with the CCR4-NOT complex in both Drosophila and mouse, it would be interesting to consider if the 3'UTR length is also affected in *DND1* mutant mouse germ cells and if DND1 is involved in regulating 3'UTR length. There is much more to understand regarding the role of DND1 and other RBPs in PGC development, but here Yamaji *et al.* have helped us to better understand the binding of DND1 to mRNAs and the CCR4-NOT complex.

Acknowledgements

Funding: None.

Footnote

Provenance and Peer Review: This article was commissioned and reviewed by the Section Editor Dr. Ziwei Li (Department of Molecular, Cell & Developmental Biology, University of California, Los Angeles, USA).

Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/amj.2017.08.07). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

References

1. Extavour CG, Akam M. Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. Development 2003;130:5869-84.
2. Williamson A, Lehmann R. Germ cell development in Drosophila. Annu Rev Cell Dev Biol 1996;12:365-91.
3. Sasaki H, Matsui Y. Epigenetic events in mammalian germ-cell development: reprogramming and beyond. Nat Rev Genet 2008;9:129-40.
4. Qi H. RNA-binding proteins in mouse male germline stem cells: a mammalian perspective. Cell Regen (Lond) 2016;5:1.
5. Jensen KB, Darnell RB. CLIP: crosslinking and immunoprecipitation of in vivo RNA targets of RNA-binding proteins. Methods Mol Biol 2008;488:85-98.
6. Yamaji M, Jishage M, Meyer C, et al. DND1 maintains germline stem cells via recruitment of the CCR4-NOT complex to target mRNAs. Nature 2017;543:568-72.
7. Noguchi T, Noguchi M. A recessive mutation (ter) causing germ cell deficiency and a high incidence of congenital testicular teratomas in 129/Sv-ter mice. J Natl Cancer Inst 1985;75:385-92.
8. Youngren KK, Coveney D, Peng X, et al. The Ter mutation in the dead end gene causes germ cell loss and testicular germ cell tumours. Nature 2005;435:360-4.
9. Northrup E, Zschemisch NH, Eisenblätter R, et al. The ter mutation in the rat DND1 gene initiates gonadal teratomas and infertility in both genders. PLoS One 2012;7:e38001.
10. Weidinger G, Stebler J, Slanchev K, et al. Dead end, a novel vertebrate germ plasm component, is required for zebrafish primordial germ cell migration and survival. Curr Biol 2003;13:1429-34.
11. Kedde M, Strasser MJ, Boldajipour B, et al. RNA-binding protein DND1 inhibits microRNA access to target mRNA. Cell 2007;131:1273-86.
12. Chen D, Wu C, Zhao S, et al. Three RNA binding proteins form a complex to promote differentiation of germline stem cell lineage in Drosophila. PLoS Genet 2014;10:e1004797.

13. Shan L, Wu C, Chen D, et al. Regulators of alternative polyadenylation operate at the transition from mitosis to meiosis. J Genet Genomics 2017;44:95-106.
14. Collart MA. The Ccr4-Not complex is a key regulator of eukaryotic gene expression. Wiley Interdiscip Rev RNA 2016;7:438-54.

doi: 10.21037/amj.2017.08.07
Cite this article as: Chen D, Hancock G. Crosslinking and immunoprecipitation: a new route for dead end. AME Med J 2017;2:119.