Crystal structure of Zucchini, an essential endoribonuclease for piRNA biogenesis

PIWI-interacting RNAs (piRNAs) are expressed in animal germlines and silence transposable elements, thereby maintaining genome integrity [1]. piRNAs are generated through two distinct biogenetic pathways: the primary processing pathway and the secondary ping-pong cycle. *Drosophila melanogaster* has three PIWI proteins (Piwi, AUB and AGO3). Piwi is expressed in ovarian somatic cells and participates in the primary pathway, whereas AUB and AGO3 are expressed in ovarian germ cells and participate in the secondary ping-pong cycle. piRNAs are initially transcribed as a long non-coding RNA precursor from repetitive genomic regions called piRNA clusters (Fig. 1). piRNA precursors are then processed into mature piRNAs by unknown nucleases, and loaded into Piwi in the primary processing pathway. Genetic and biochemical studies revealed that a number of proteins are involved in the primary processing pathway. These include the putative RNA helicase Armitage, and the Tudor domain-containing RNA helicase Yb, which are both localized in Yb bodies, cytoplasmic perinuclear non-membranous organelles, and the putative nuclease Zucchini (Zuc), which are localized on the outer surface of mitochondria.

Zuc consists of an N-terminal mitochondrial localization sequence, a putative transmembrane helix, and a catalytic domain belonging to the phospholipase D (PLD) superfamily, which is characterized by the His-Lys-Asp (HKD) motif. The PLD superfamily members cleave the phosphodiester bond in different substrates, thereby participating in a variety of cellular processes. The PLD superfamily includes a number of enzymes with different substrate specificity, such as PLD, which degrades lipid substrates, and the bacterial nuclease Nuc, which degrades nucleic acid substrates. Zuc shares the highest sequence similarity with Nuc among the PLD superfamily members, suggesting that Zuc may be a ribonuclease involved in piRNA biogenesis. However, previous studies failed to detect the nuclease activity of Zuc. Thus, the molecular identity of a piRNA processing ribonuclease remains elusive, and the molecular mechanism by which Zuc is involved in primary piRNA biogenesis remains unknown.

To understand Zuc's function, we set out to solve the crystal structure of *D. melanogaster* Zuc. The cytoplasmic region of Zuc was expressed in *Escherichia coli*, purified, and crystallized [2]. X-ray diffraction data were collected at beamline **BL32XU**. We solved the crystal structure of Zuc at 1.75 Å resolution by the MAD method using a selenomethionine-labeled protein [3]. The crystal structure revealed that the cytoplasmic region of Zuc consists of a catalytic domain and a zinc-binding domain (Fig. 2(a)). Zuc forms a dimer, in which His169 and Lys171 in the HKD motif in the two molecules create an active-site groove at the dimer interface. The catalytic domain consists of an eight-stranded mixed β-sheet flanked by α-helices on both sides, and shares structural similarity with the other PLD superfamily members. Secondary structure prediction suggested that, unlike other PLD superfamily members, Zuc may have a disordered region between the first β-strand and α-helix in the catalytic domain. The crystal structure revealed that this region unexpectedly forms the zinc-binding domain, in which

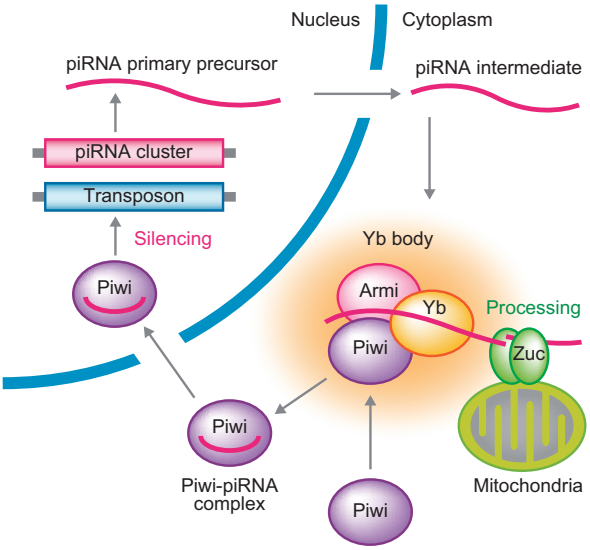

Fig. 1. Model of primary piRNA biogenesis in *D. melanogaster*.

a zinc ion is coordinated by three cysteine residues and one histidine residue. A structural comparison of Zuc with Nuc revealed a notable difference in their active-site grooves. Nuc has a wide, positively charged groove that can accommodate double-stranded nucleic acids (Fig. 2(b)), which is consistent with the data showing that Nuc cleaves both a single-stranded RNA (ssRNA) and a double-stranded RNA (dsRNA). In contrast, Zuc has a narrow catalytic groove, which appears to accommodate ssRNA but not dsRNA (Fig. 2(a)). Zuc has a positively charged surface on the side opposite to the active site, where the N termini of the two molecules are located, which is consistent with the previous data showing that Zuc is localized on the outer mitochondrial membrane. Together, our structural analysis indicated that Zuc is a single-strand-specific nuclease that functions on the mitochondrial surface.

We examined the nuclease activity of Zuc by *in vitro* cleavage assays using radiolabelled synthetic RNAs, and found that Zuc cleaved ssRNA substrates in a non-sequence-specific manner. The mutant Zuc proteins, including H169A and K171A, failed to cleave the ssRNA substrate, confirming the functional significance of the active-site groove. The Zuc mutant lacking the zinc-binding domain showed reduced ssRNase activity, indicating the involvement of the zinc-binding domain in ssRNA binding. Zuc cleaved ssRNA but not dsRNA, indicating that Zuc is a single-strand-specific nuclease. Zuc also cleaved circular ssRNA, indicating that Zuc is an endonuclease. MmZuc also showed ssRNase activity, confirming that Zuc is an evolutionarily conserved nuclease. The cleavage products possess a 5' monophosphate, a hallmark of mature piRNAs bound to Piwi. We further examined the biological relevance of the nuclease activity of Zuc for transposon silencing by plasmid rescue experiments in ovarian somatic cells. We found that wild-type Zuc, but not the active-site mutants showing no ssRNase activity, rescued transposon derepression, indicating that the nuclease activity of Zuc is critical for transposon silencing *in vivo*. Together, our functional analysis revealed that Zuc is a single-stranded-specific endoribonuclease that defines the 5' end of mature piRNAs. We thus propose a model for primary piRNA biogenesis in animal germlines, in which Zuc acts as an endoribonuclease in primary piRNA maturation (Fig. 1).

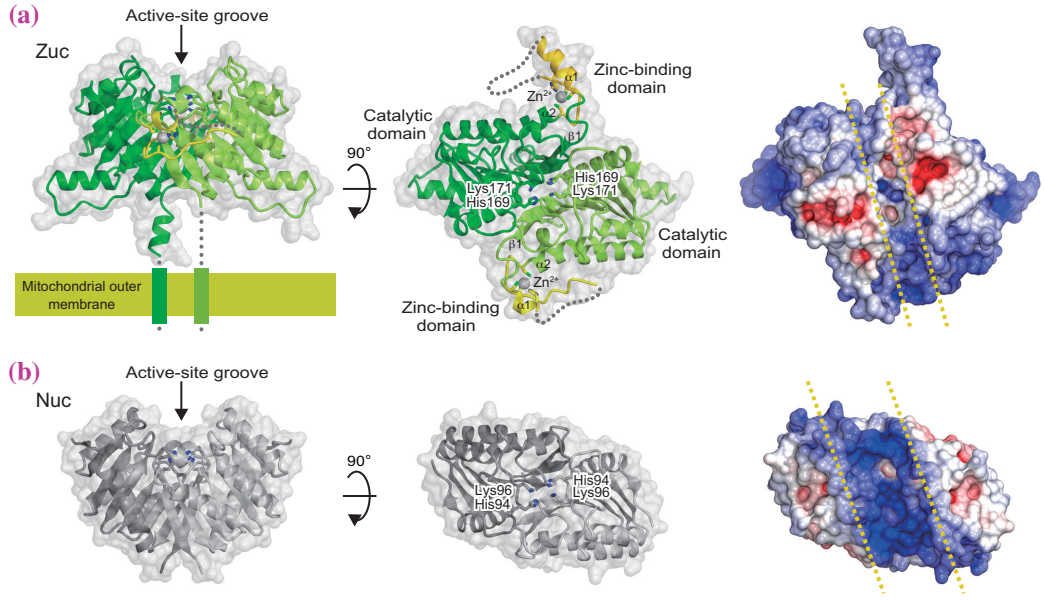

Fig. 2. **(a)** Crystal structure of Zuc. **(b)** Crystal structure of Nuc (PDB: 1BYR). Yellow dashed lines indicate the positively charged active-site groove.

Hiroshi Nishimasu* and Osamu Nureki

Department of Biophysics and Biochemistry, The University of Tokyo

*Email: nisimasu@biochem.s.u-tokyo.ac.jp

References

[1] M.C. Siomi *et al.*: Nat. Rev. Mol. Cell. Biol. **12** (2011) 246.
[2] S. Fukuhara *et al.*: Acta Crystallogr. F Struct. Biol. Cryst. Commun. **68** (2012) 1346.
[3] *H. Nishimasu, H. Ishizu, K. Saito, S. Fukuhara, M. K. Kamatani, L. Bonnefond, N. Matsumoto, T. Nishizawa, K. Nakanaga, J. Aoki, R. Ishitani, H. Siomi, M. C. Siomi, O. Nureki: Nature* ***491*** *(2012) 284.*