An Integrated Analysis Combined with Automated X-Ray Crystallography for an Insect Ecdysteroidogenic Enzyme, Noppera-Bo

Noppera-bo (Nobo) is an insect-specific glutathione S-transferase that is involved in the biosynthesis of ecdysteroids, which play a pivotal role in insect development. We determined the crystal structures of Nobo from *Drosophila melanogaster* (DmNobo) in complex with its inhibitors using a fully-automated X-ray data collection system. We performed *in silico, in vitro*, and *in vivo* analyses based on the crystal structures of the DmNobo–inhibitor complexes. Our structure-based integrated analysis revealed that Asp113 is a crucial residue for the Nobo function and is a suitable target for Nobo inhibitors.

The *noppera-bo* (*nobo*) is one of the Halloween genes responsible for ecdysteroid biosynthesis [1]. As ecdysteroids are involved in insect development, the Nobo protein can be a suitable target for insecticides. The deletion mutant of *nobo* is embryonic-lethal. DmNobo, the Nobo protein from *Drosophila melanogaster*, is a member of the epsilon class of cytosolic glutathione *S*-transferases (GST, EC 2.5.1.18). While DmNobo has enzymatic GST activities, an endogenous substrate has not been identified. Therefore, we developed an artificial substrate for DmNobo and screened its possible enzymatic activity inhibitors using a chemical compound library at the University of Tokyo. The screening identified 12 compounds, including 17β-estradiol, with inhibitory activity against DmNobo [2, 3, 4]. To analyze the inhibition mechanism of Nobo, we conducted a crystal structure analysis of DmNobo; no tertiary structures of the Nobo proteins were obtained.

We determined the crystal structures of DmNobo in glutathione (GSH)-free and -complex forms at 1.50 and 1.75 Å resolutions, respectively [3]. The crystal structures demonstrated that DmNobo forms a homodimer with a canonical GST fold and has a well-conserved GSH-binding site (G-site) and a hydrophobic substrate-binding site (H-site) adjacent to the G-site. Next, we tried to obtain the crystal structures of DmNobo–GSH–inhibitor complexes using the soaking method with the identified inhibitors. However, it was difficult to determine suitable conditions for the soaking experiments; we expected that many trial-and-error experiments would be required to optimize the soaking conditions. Therefore, we used PReMo, a fully-automated X-ray data collection and processing system developed by the Structural Biology Research Center (SBRC). In addition, we developed an automated structural analysis software pipeline, PEINTS (https://github.com/KotaroKoiwai/PEINTS), which performs molecular replacement phasing and crystallographic refinement and displays a difference Fourier map around the active site using the programs MOLREP, REFMAC5, and COOT, respectively (**Fig. 1**) [4]. This system enabled us to optimize the soaking conditions through quick data collection and crystal structure determination, obtaining seven inhibitor-complex structures at a 1.40–1.84 Å resolution (**Fig. 2A**) [3, 4].

Based on the crystal structures of DmNobo–GSH–inhibitor complexes, we performed *in silico*, *in vitro*, and *in vivo* analyses, revealing that Asp113 is essential for the biochemical and biological functions of DmNobo (**Fig. 2B, E, F, G**) [3]. The crystal structure of the DmNobo–GSH–17β-estradiol complex showed that 17β-estradiol occupies the H-site. The binding of 17β-estradiol induces a rotation of the χ1 angle of Asp113 by 25.4° to form a hydrogen bond with 17β-estradiol (**Fig. 2C**). A fragment molecular orbital (FMO) calculation estimated that the hydrogen bond contributed –41.4 kcal/mol in the total interaction energy of –82.4 kcal/mol for 17β-estradiol (approximately 50% of the total interaction energy). A molecular dynamics

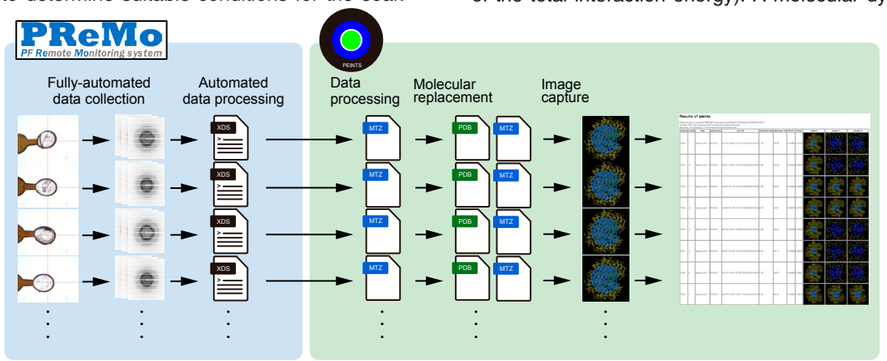

Figure 1: A fully-automated data collection system, PReMo, and a data processing pipeline, PEINTS.

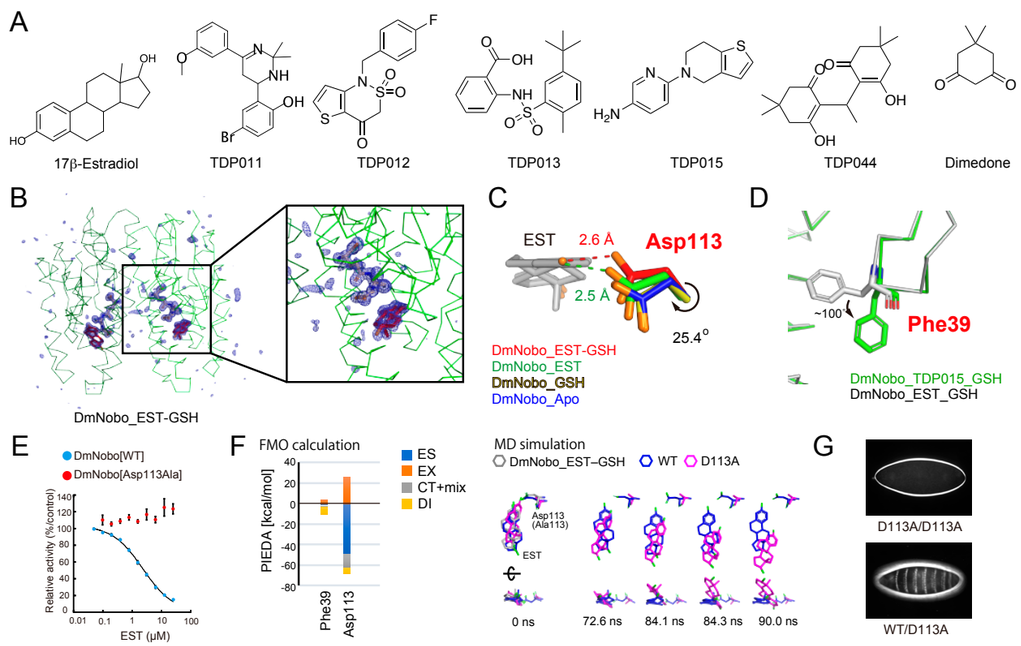

Figure 2: An integrated analysis for Nobo: **A** Chemical structures of DmNobo inhibitors used in this study. **B** Crystal structure of DmNobo in complex with 17β-estradiol and GSH (DmNobo_EST-GSH). **C** Conformational change of Asp113 by binding with 17β-estradiol. **D** Conformational change of Phe39 by binding with TDP015. **E** *In vitro* functional analysis of Asp113 with DmNobo_D113A mutant shown by an enzymatic assay. **F** FMO calculation (left) and MD simulation (right) for Asp113. **G** *In vivo* analysis of Asp113. Figures are reproduced from references 3 and 4.

(MD) simulation also suggested that the hydrogen bond is essential for stable binding between DmNobo and 17β-estradiol. The 17β-estradiol did not inhibit the enzymatic activity of an Ala-substituted variant at Asp113 (DmNobo_D113A) (**Fig. 2E**). These experimental and *in silico* analyses demonstrated a pivotal role for Asp113 in the biochemical function of DmNobo. In addition to Asp113, we also found Phe39 contributes to ligand recognition (**Fig. 2D**).

Next, we investigated the biological significance of Asp113. Phylogenetic analysis revealed that Nobo proteins were found in Diptera and Lepidoptera. The Nobo proteins form a sub-family (the Nobo family), and Asp113 is highly conserved among the Nobo proteins, suggesting its biological significance. We performed a genetic analysis to confirm its biological function. The homozygous nobo allele with the D113A mutation exhibited embryonic lethality and an undifferentiated cuticle structure, a phenocopy of complete loss-of-function *nobo* homozygotes (**Fig. 2G**). These results suggest that the Nobo proteins acquired Asp113 for their biological functions. The Asp residue may be essential for binding an endogenous sterol substrate to regulate ecdysteroid biosynthesis.

Our study revealed the first crystal structure of insect steroidogenic Halloween proteins. The structure-based integrated analysis clarified the biological significance of Asp113 in DmNobo. In addition, Asp113 is a critical target for developing insecticides that specifically inhibit ecdysteroid biosynthesis. We would like to emphasize that highly automated X-ray crystallography played an essential role in our study. The high-throughput analysis enabled us to perform intensive optimization of soaking experiments, leading to the success of integrated research, including *in vivo* analysis.

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BEAMLINES

BL-1A, BL-5A, BL-17A and AR-NE3A

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