IGTRCN Peer-to-peer training program report

Induction of homeotic wing transformations in a pyralid moth using CRISPR/Cas9 mutagenesis

Trainee: Arnaud Martin
Post-Doctoral Research Scholar in the Laboratory of Dr. Nipam Patel.
Department of Molecular Cell Biology, University of California, Berkeley, California, USA.

Host PI: Paul D. Shirk
Center for Medical, Agricultural and Veterinary Entomology, Agricultural Research Service, US Department of Agriculture, Gainesville, Florida, USA

Summary: The *Hox* gene *Ultrabithorax* (*Ubx*) is notorious for establishing the distinct morphologies of wings vs halteres and wings vs. elytrae in Diptera and Coleoptera. Here we used CRISPR/Cas9 somatic mutagenesis to generate *Ubx*-deficient cell clones in Lepidoptera, using the wings of the pyralid moth *Plodia interpunctella*. Following syncytial embryo injection of Cas9 + *Ubx* sgRNA within 160 minutes after egg laying, 25% of individuals reached the adult stage, among which 18% displayed hindwing-to-forewing homeotic transformations consistent with the expected role of *Ubx* in Lepidoptera. These results suggest that the CRISPR methodology and the *Plodia* system combine as a strategy of choice for routine functional genomics in Lepidoptera.

The wing patterns of butterflies and moths form a pinnacle of morphological variation but are currently limited by a lack of practical tools allowing the functional characterization of the genes involved in patterning. In order to "unleash" the potential of this clade in developmental genetics, it is necessary to establish a powerful model system for functional work in Lepidoptera, comparable to *Tribolium* and *Drosophila*, and easier to maintain than silkworms, that are classically used for lepidopteran genetics. Other requirements for a tractable model organism include the ease to share lines between laboratories, resistance to inbreeding and laboratory conditions, inducible egg production, and availability of genomic resources. The Indian Mealmoth (*Plodia interpunctella*) fulfills these criteria and has been maintained for 25 years in the laboratory of Dr. Paul Shirk (USDA, Gainesville FL). The goals of my visit in the Shirk lab were: **(1)** to learn from the Shirk lab the rearing and egg injection protocols relevant to future genetic work with *Plodia* (Bossin et al., 2007); **(2)** to assess the feasibility of CRISPR/Cas9 targeted mutagenesis in this moth (Gilles and Averof, 2014); and **(3)** to generate mutant pattern phenotypes on adult wings. In a larger perspective, the training funded by the IGTRCN aimed at developing tools that will benefit the work of other groups, including in agronomical research. For strategic reasons, I focused on modification of the soma only, but will extend this work to germline transformation in the near future.

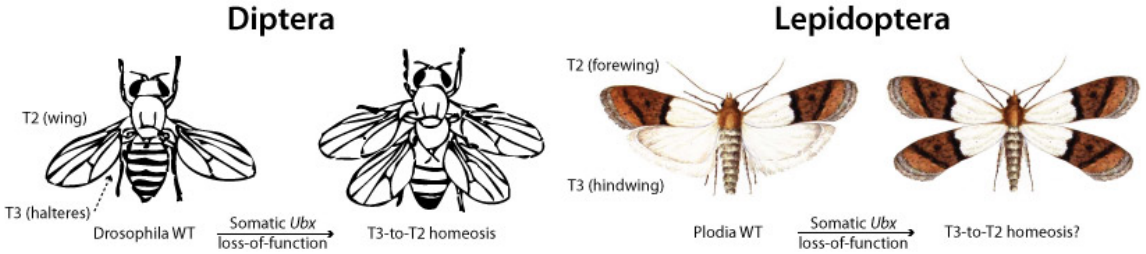
Plodia: a suitable lepidopteran laboratory species for genome editing

The rearing of *P. interpunctella* moths is straightforward and takes a minimal amount of resources and manipulation time. The life cycle is completed in about 26 days in a temperature incubator set at 28°C. Eggs are dispatched in containers containing a mix of wheat bran, glycerol and honey, and develop until the adult stage without human intervention.

The day of the injections, adults are anaesthetized with CO_2 and transferred to an egg laying jar. The gas rapidly triggers massive release of freshly fertilized eggs which can be collected at regular intervals for immediate injection. The injection procedure consists in orienting and aligning eggs on a cellophane strip, injecting the posterior end using a microscope set-up, and sealing with glue. A single, well-trained operator can inject 400 eggs within 2h after egg laying (AEL), and if injecting embryos as early as possible is critical (*e.g.* for germ line targeting or to limit somatic mosaicism), a duo of operators can coordinate to continuously inject eggs within 30-40 min AEL for several hours (about 800 eggs for a 3h session).

Of note, *P. interpunctella* genomic resources are rising: a published transcriptome can already be used for isolation of candidate transcripts (Harrison et al., 2012), and an unpublished genome assembly is currently available upon request as of April 2015 (Dr. Steve Paterson, University of Liverpool).

Ubx specifies the identity of wing-derived structures in insects

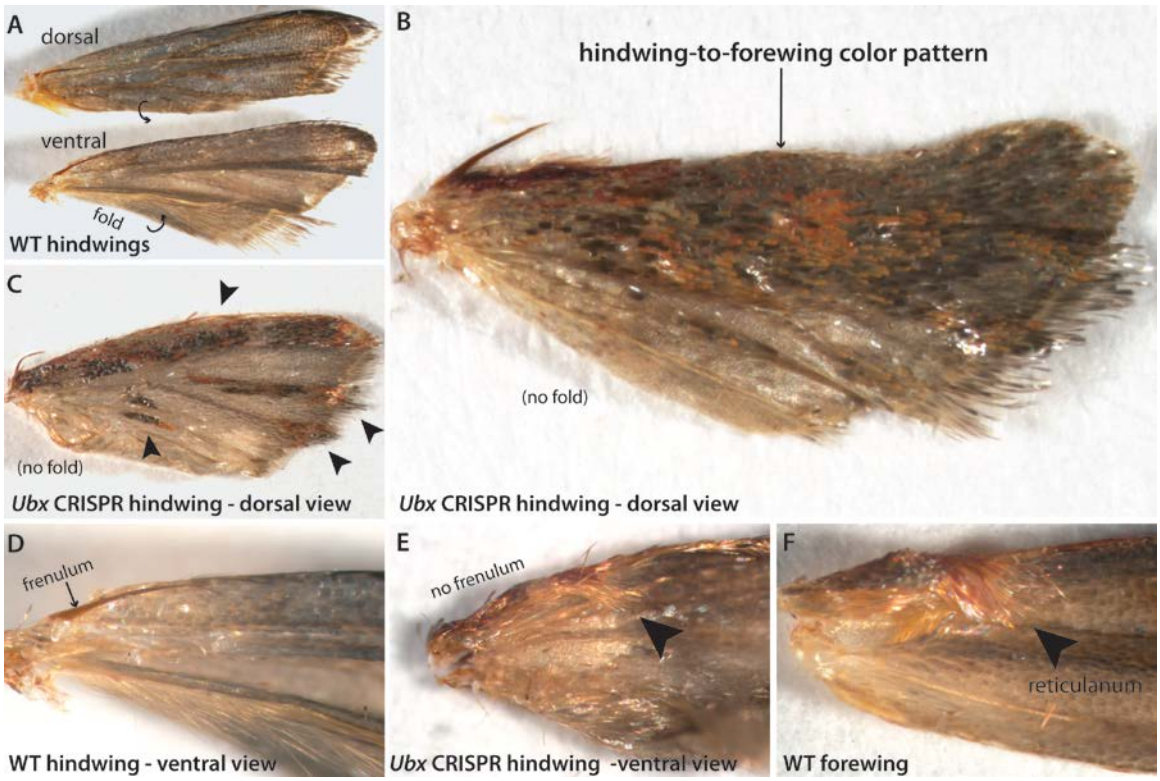


Somatic disruption of the *Ubx* gene in in Diptera and Coleoptera results in segment identity defects that transform the T3 segment into a T2 segment (Tomoyasu et al., 2005; Weatherbee et al., 1998). A reverse experiment has produced complementary results in Lepidoptera, with ectopic expression of *Ubx* in butterfly T2 forewings resulting in homeotic T3-like or "forewing-to-hindwing" color pattern shifts (Lewis et al., 1999). *Ubx* expression is restricted to the T3 segment in Diptera, Coleoptera and Lepidoptera, and we can conclude that its function – the repression of T2-like identity in the T3 segments - is conserved among these three lineages . Based on this assumption, it is predicted that *Ubx* loss-of-function experiments in *P. interpunctella* should generate forewing-like features on hindwings, such as brown/black pigmentation or wing shape defects.

CRISPR/Cas9 mutagenesis of the Ubx gene in Plodia

To test this hypothesis, I injected a mixture of Cas9 protein (supplier: PNA Bio) and *P. interpunctella Ubx* sgRNA into 709 syncytial embryos timed between 100 and 160min AEL. 43% (N=306) of the injected eggs hatched, and we recovered a total of 182 adults after 30 days. All adults were examined individually after removal of the forewing, revealing 34 individuals (18%) with hindwing cell clones showing a forewing homeosis. This number is not indicative of the efficiency of CRISPR, because mutant animals with large Ubx-deficient clones are not expected to reach the adult age and have not been monitored in our assay. Perhaps for a similar reason, no individual showed a complete hindwing-to-forewing transformation. Rather, aberrant hindwings where characterized by large patches of pigmented scales characteristic to the WT forewing (**panels A-C**). In addition, mutant hindwings usually lacked their usual posterior fold and appeared more rigid, here again indicative of a hindwing-to-forewing mosaic homeosis. Of note, no individual showed mutant clones on both its left and right wings, illustrating the principle that G_0 phenotypes reflect somatic mosaicism.

Finally, at least two individuals lacked the hindwing-specific frenulum, a hook-like modified bristle that is used in wing attachment, and developed modified scales reminiscent of the forewing-specific reticulanum, which serves as a furrow for the frenulum (**panels D-F)**. Together, these results validate the hypothesis that *Ubx* inhibits forewing-like identity in the hindwing, and also establish CRISPR/Cas9 mutagenesis as a valuable tool for generating mutant phenotypes in *Plodia* adult wings.



Main Figure – Ubx CRISPR induces homeotic hindwing-to-forewing mosaicism in *P. interpunctella.* A: Dorsal and ventral views of wild-type hindwings, characterized by a posterior fan-like, folded structure, and the absence of black-brown (forewing specific scales). **B-C:** *Ubx* CRISPR induces mosaic transformations of the hindwings into forewings, as seen by the presence of forewing-like color scales and by the loss of the posterior fold. In contrast with Panel B, Panel C shows many small mutant clones (arrowheads). **D-F** In rare instances, *Ubx* CRISPR induces loss of the hindwing-specific frenulum involved in attaching the hindwing to the forewing frenulum. In such transformants, the base of the hindwing displays scale structures evocative of a forewing identity (pink scales reminiscent of the reticulanum, arrowheads)

Conclusions

The IGTRCN Peer-to-peer program offered me the opportunity to discover a new model system for my research on wing patterning, and the Shirk Lab was perfectly suited for this training. I was particularly impressed by the experimental potential of *Plodia* for developmental genetics, and my preliminary results demonstrated the suitability of CRISPR targeted mutagenesis to generate somatic loss-of-function phenotypes in adult structures. Future experiments with this system will consist in optimizing transformation efficiency and generating stable mutant lines.

Acknowledgements

I am immensely grateful to the IGTRCN for providing the opportunity to do this work, Paul Shirk for his enthusiasm, support and expertise, and Richard Furlong for technical assistance in the Shirk lab.

References

Bossin, H., Furlong, R.B., Gillett, J.L., Bergoin, M., and Shirk, P.D. (2007). Somatic transformation efficiencies and expression patterns using the JcDNV and piggyBac transposon gene vectors in insects. Insect Mol. Biol. *16*, 37–47.

Gilles, A., and Averof, M. (2014). Functional genetics for all: engineered nucleases, CRISPR and the gene editing revolution. EvoDevo *5*, 43.

Harrison, P.W., Mank, J.E., and Wedell, N. (2012). Incomplete Sex Chromosome Dosage Compensation in the Indian Meal Moth, Plodia interpunctella, Based on De Novo Transcriptome Assembly. Genome Biol. Evol. *4*, 1118–1126.

Lewis, D.L., DeCamillis, M.A., Brunetti, C.R., Halder, G., Kassner, V.A., Selegue, J.E., Higgs, S., and Carroll, S.B. (1999). Ectopic gene expression and homeotic transformations in arthropods using recombinant Sindbis viruses. Curr. Biol. *9*, 1279–1287.

Tomoyasu, Y., Wheeler, S.R., and Denell, R.E. (2005). Ultrabithorax is required for membranous wing identity in the beetle Tribolium castaneum. Nature *433*, 643–647.

Weatherbee, S.D., Halder, G., Kim, J., Hudson, A., and Carroll, S. (1998). Ultrabithorax regulates genes at several levels of the wing-patterning hierarchy to shape the development of the Drosophila haltere. Genes Dev. *12*, 1474–1482.