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## Ouija board: A transcription factor evolved for only one target in steroid hormone biosynthesis in the fruit fly *Drosophila melanogaster*

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### ABSTRACT

Transcription factors generally regulate gene expression of multiple targets. In contrast, our recent finding suggests that the zinc finger protein Ouija board controls steroid hormone biosynthesis through specific regulation of only one gene *spookier* in *Drosophila*. It sheds light on a specialized but essential factor that evolved for one target.

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### Introduction

In the context of developmental programs, a variety of gene cascades are regulated by a number of transcription factors (TFs). To accomplish the precise morphogenesis and tissue organization, it is important to regulate gene expression profiles appropriately in space and time. In this sense, the expression of TFs should be also tightly controlled in spatiotemporal-dependent manners.<sup>1</sup>

Some TFs are known as the “master” regulators that are critical for tissue morphogenesis and pattern formation. For example, Hox genes control a set of downstream genes that produce tissues and organs along the body axes.<sup>2</sup> Another striking example is Eyeless/Pax6, which serves as a master TF for eye morphogenesis.<sup>2</sup> Such master TFs are usually highly conserved among animal phyla, and the transcription of multiple components for a particular developmental process can be cooperatively regulated in a wide variety of animals.

While most TFs, including the master TFs, usually bind multiple sites in the genome to regulate a large number of target genes, less is known about the specific TFs that regulate only few or even one specific

target(s). It also remains to be determined whether such specialized TFs are essential in development.

We have recently identified a new TF designated Ouija board (Ouib) essential for steroid hormone biosynthesis in the fruit fly *Drosophila melanogaster*.<sup>3</sup> It is predominantly expressed in the steroidogenic organs and, as far as we know, represents the first example of a TF that appears to be specialized for inducing the expression of only one steroidogenic enzyme gene. In this review, we discuss a role of Ouib and its biological significance for development and evolution.

### Transcriptional regulation of the steroid hormone biosynthesis pathway

Steroid hormones have pleiotropic actions on cells, controlling development, homeostasis, and reproduction. They are derivatives of cholesterol and other suitable sterols, being biosynthesized in specialized endocrine organs, such as the adrenal gland and gonads in vertebrates. In the biosynthesis pathway, a battery of steroidogenic enzymes, including cytochrome P450 family, is required to convert suitable sterols into steroid hormones through several intermediate multiple steps. These enzyme genes

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are specifically expressed in the steroidogenic organs. Therefore, the high activity of steroid hormone biosynthesis is achieved by the high transcriptional activity of these steroidogenic genes.

Vertebrate steroidogenic genes are coordinately regulated by a TF called NR5R1/Steroidogenic factor-1 (SF-1).<sup>4</sup> SF-1 is predominantly expressed in steroidogenic organs. SF-1 knockout mice lose gonads and adrenal glands, and die shortly after birth.<sup>5,6</sup> Moreover, the expression of SF-1 is sufficient for the differentiation of non-steroidogenic stem cells into steroidogenic cells by inducing the expression of steroidogenic enzyme genes.<sup>7</sup> In this sense, vertebrate steroidogenesis depends on the transcriptional activity of SF-1, and as such, SF-1 is a master regulator for the development of gonads and adrenal glands as well as for steroidogenesis following organogenesis.

SF-1 genes are well conserved in animal evolution. Nevertheless, it seems that the function of SF-1 may differ between vertebrates and invertebrates, as an insect ortholog of SF-1, Ftz-F1, is highly expressed not only in steroidogenic organs but also in many other tissues.<sup>8</sup> A previous study has argued that the steroid hormone biosynthesis pathway and its transcriptional regulatory mechanism have separately evolved in insects,<sup>9</sup> and how to transcriptionally regulate steroidogenic enzyme genes has not been known until recently.

### The biosynthesis of insect steroid hormone, ecdysteroid

Insect steroid hormones, ecdysteroids, control the timing of developmental progression, such as molting, metamorphosis, and reproductive maturation.<sup>1,10-12</sup> The last decade is a very fruitful period in the biology of ecdysteroids: several research groups including our group have successfully identified and characterized a number of genes essential for the ecdysteroid biosynthesis pathway.<sup>10</sup> Among the identified genes, *neverland* (*nvd*), *spook* (*spo*), *spookier* (*spok*), *Cyp6t3*, *shroud* (*sro*), *phantom* (*phm*), *disembodied* (*dib*), *shadow* (*sad*), and *shade* (*shd*) encode ecdysteroidogenic enzymes that catalyze the conversion steps from suitable sterol to 20-hydroxyecdysone and/or other ecdysteroids (Fig. 1). These enzyme genes, except for *shd*, are predominantly expressed in a specific endocrine organ called the prothoracic gland (PG). The expression levels of these genes fluctuate during developmental stages and well correlated to the ecdysteroid titer, indicating that their

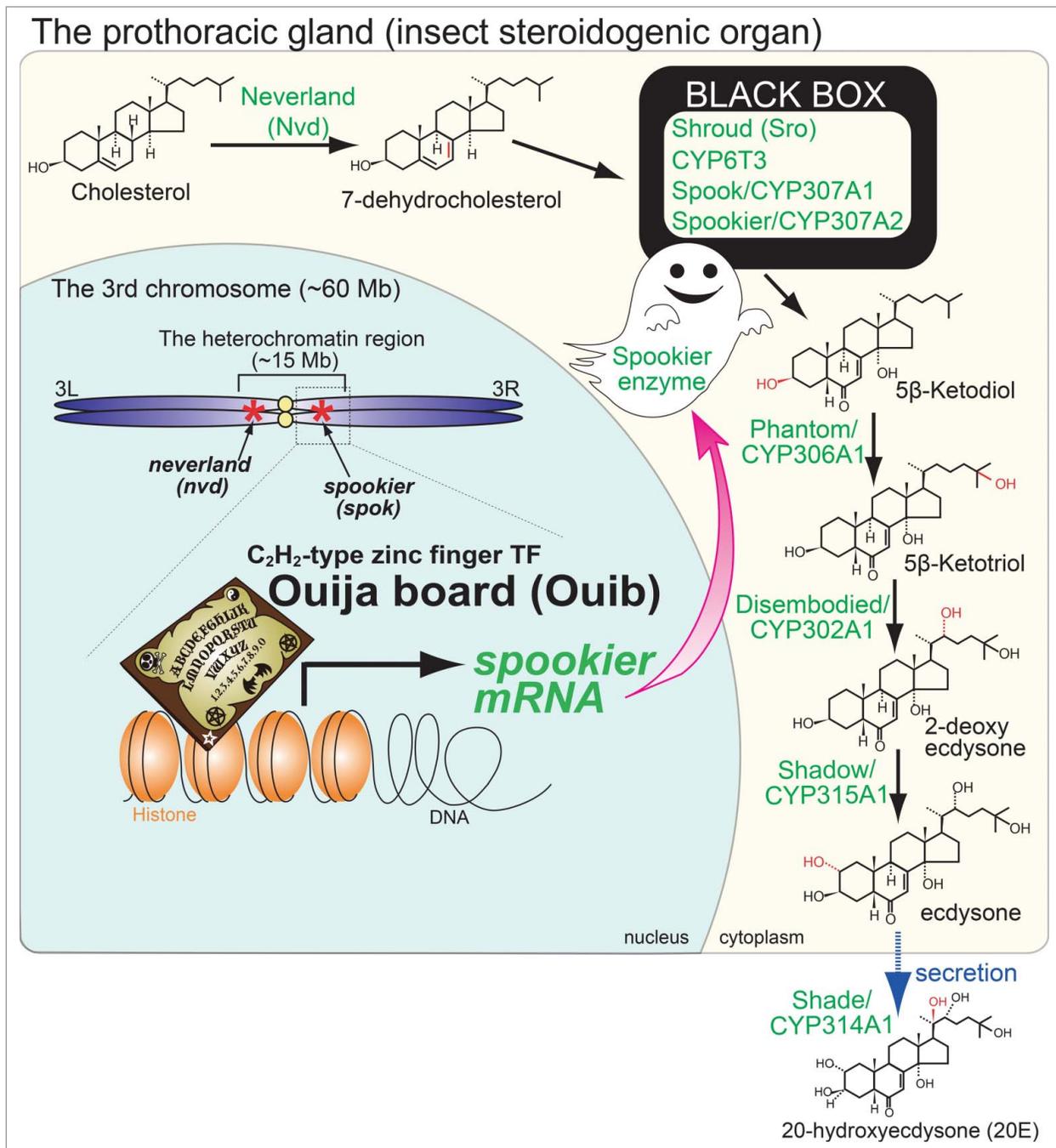
transcriptional activities are tightly regulated.<sup>13-15</sup> Therefore, the spatiotemporal regulation of ecdysteroidogenic genes is essential for ecdysteroid biosynthesis. This is also the case with steroidogenesis in vertebrates.

A number of ecdysteroidogenic TFs have also been reported. The first description of a steroidogenic TF was the discovery that  $\beta$ FTZ-F1 plays a role in expression of *phm*, *dib*, and *sad*.<sup>16</sup>  $\beta$ FTZ-F1 is an isoform of FTZ-F1, being transcribed from late-stage embryos to adults.<sup>17,18</sup> Since the discovery of  $\beta$ FTZ-F1, the list of ecdysteroidogenic transcription factors has grown subsequently until 2014, including Molting defective (*Mld*), *DHR4*, *Broad*, and *Ventral vein lacking* (*Vvl*).<sup>1</sup> Notably, all of these TFs are expressed not only in the PG, but also in other type of cells. Therefore, these TFs *per se* do not account for the spatial restriction of ecdysteroidogenic gene expression. It is likely that a combination of TFs can provide the PG-specific expression, along with epigenetic programming. On the other hand, it is also theoretically possible that there are unknown TFs whose expression and function are restricted to the PG.

### *Ouija board* encodes the C<sub>2</sub>H<sub>2</sub>-type zinc finger type TF and is predominantly expressed in the ecdysteroid-producing organ, the prothoracic gland

In 2015, we reported a C<sub>2</sub>H<sub>2</sub>-type zinc finger protein gene *CG11762*, and named it *ouija board* (the reason is explained below).<sup>3</sup> *ouib* is predominantly expressed in the PG and *Ouib* protein is localized in the nuclei of the PG cells (Takemata, Kamiyama, YSN, and RN, unpublished observation). *ouib* null mutants arrest development in the 1st instar stage and the ecdysteroid titer is significantly reduced. Ecdysone signaling is also significantly reduced in these mutants, suggesting that *Ouib* is essential for development and involved in ecdysteroid biosynthesis. Considering the predicted primary structure of *Ouib*, we expected that *Ouib* might act as a TF that regulates the expressions of ecdysteroidogenic genes

To examine which step of the ecdysteroid biosynthesis pathway is regulated by *Ouib*, we measured the expression levels of ecdysteroidogenic genes in *ouib* null mutant larvae. Among six tested, the expression levels of *spok*, *dib*, and *sad* were significantly reduced. In particular, *spok* expression is drastically reduced as *Spok* protein level is almost



**Figure 1.** The ecdysteroid biosynthesis pathway and the role of Ouija board on *spookier* expression in the fruit fly *Drosophila melanogaster*. In the prothoracic gland (insect steroidogenic organ), cholesterol and other sterols are converted into ecdysteroids via several intermediate steps. The modifications on carbon residues and the responsible enzymes are indicated with red and green, respectively. In the nucleus, *nvd* and *spok* are located in the heterochromatin region. A zinc finger transcription factor named “Ouija board (Ouib)” specifically binds to the promoter region of “*spookier*” and induces expression of this gene. Both Ouib and Spok are essential for ecdysteroid biosynthesis during larval development.

abolished in the PG of *ouib* mutant larvae. Moreover, the qRT-PCR analysis revealed that *spok* expression temporally correlates well with *ouib* expression during the larval-to-pupal transition. These results indicate that *ouib* is required for *spok* expression in the PG.

### Ouija board is specifically required for the expression of *spookier* in the ecdysteroid biosynthesis pathway

We have also conducted *in vitro* reporter assays for Ouib transcriptional activity on *spok*, and found that a

critical sequence of 15 bp within 300 bp of the *spok* promoter region is sufficient for Ouib-dependent gene expression in cultured cells. Furthermore, our EMSA experiment has confirmed the direct binding of Ouib Zinc finger domain to the 15 bp region.

To address how specific *ouib* is for regulating *spok* expression, we performed ecdysteroid-feeding rescue experiments. We put *ouib* mutant larvae on food supplemented with 20-hydroxyecdysone or several upstream precursors. Indeed, the larval arrest phenotype of *ouib* mutant larvae is rescued by administration of 5 $\beta$ -ketodiol and 20E, but not by cholesterol or 7-dehydrocholesterol. This is consistent with the hypothesized catalytic step mediated by the enzyme Spok, as this is known to act in the conversion step from 7-dehydrocholesterol to 5 $\beta$ -ketodiol (Fig. 1). Furthermore, we have performed the rescue experiments by transgenic expression. Surprisingly, overexpression of the equivalent isoform of *spok* almost fully restores the developmental arrest phenotype of *ouib* mutants and they grew up to the adult stage. These results suggest that the function of Ouib mainly dedicates to *spok* expression.

In general, a transcription factor has multiple target genes. In contrast, our data suggest that this is not always the case. All of our data strongly support the idea that Ouib is essential for development via mediating a transcriptional activation of only one ecdysteroidogenic enzyme Spok. Because this zinc finger TF can induce the expression of the gene “*spookier*,” we named its TF after “Ouija board,” which was an instrument believed to be used in Western culture to attempt communication with the dead (Fig. 1).

### The “catalytic step-specific” transcriptional regulation for steroid hormone biosynthesis

Ouib is the first invertebrate TF specialized for steroid hormone biosynthesis. Moreover, it provides the first example of the “catalytic-step specific” transcriptional regulation for steroid hormone biosynthesis. While all of the previously known steroidogenic TFs regulate multiple enzyme genes in insects as well as mammals, our study sheds light on a specialized TF that has been evolved only for one target gene in a specific catalytic step. Although we cannot completely rule out the possibility that Ouib is also involved in direct transcriptional regulation of genes other than *spok*, particularly *dib* and *sad*, further studies are required to clarify the

extent to which Ouib regulates other genes, and the functional importance of these genes, through a transcriptome analysis/ChIP-seq analysis together with eventual mutational analysis of any identified targets.

What is the biological significance of such catalytic-step specific regulations at the transcription level? It should be noted that Spo/Spok act in the catalytic step referred to as “BLACK BOX,” where the intermediates from 7-dC to 5 $\beta$ -ketodiol are presumably unstable so that they have not fully been identified.<sup>19</sup> Considering that the “BLACK BOX” has been thought to act as the rate-limiting step in the ecdysteroid biosynthesis pathway,<sup>20</sup> it raises an interesting possibility that this bottleneck for ecdysteroid biosynthesis depends on catalytic-step specific transcriptional regulation. Identification of the substrates of Spo/Spok would lead to characterization of the reaction steps in the “BLACK BOX.”

### A transcriptional regulation of ecdysteroidogenic genes located in the heterochromatin region

Previous studies reported that the expression of *spok* is regulated by another zinc finger protein Mld.<sup>19,21,22</sup> Therefore, the transcription of *spok* is dependent on the interaction between at least two zinc-finger proteins, Ouib and Mld. In contrast to Ouib, Mld appears not to be specific for the regulation of *spok* expression. First, Mld, unlike Ouib, is expressed in several other tissues during development besides the PG.<sup>21</sup> Second, the *mld* loss-of-function phenotype is not rescued by overexpressing either *spo* or *spok*.<sup>19</sup> Third, consistent with results of the transgenic experiment, Mld is essential for regulating expression not only *spok* but also another ecdysteroidogenic genes such as *nvd*.<sup>22</sup> Therefore, Ouib and Mld overlap in their regulation of *spok* expression, but also have distinct functions during development. It would be intriguing to examine a functional relationship between Ouib and Mld for induction of *spok* expression in the PG.

It is of interest to note that the gene loci of *spok*, as well as *nvd*, are located in the pericentric heterochromatin region on the 3rd chromosome of *D. melanogaster* (Fig. 1). It is therefore feasible to consider that some chromatin remodeling would be necessary to regulate the expression of *spok* and *nvd*. Consistent with this notion, it has been reported that dATAC histone acetylase complex and the insulator

protein CTCF regulates ecdysteroidogenic gene expression.<sup>23-25</sup> However, to our knowledge, it is largely unknown how the heterochromatin region is decondensed to allow TFs to bind to the promoter region for inducing gene expression. We assume that there is a regulatory mechanism by which Mld and Ouib temporally decondense the heterochromatin status of *nvd* locus and/or *spok* locus. Such a heterochromatin remodeling system may contribute to the spatiotemporal dynamics of ecdysteroid biosynthesis by restricting the expression of *nvd* and *spok*. In any case, the interactions between chromatin status and transcriptional activity should be tightly regulated in the earlier steps of ecdysteroid biosynthesis.

### Evolutionarily divergence of transcriptional regulation in steroid hormone biosynthesis

Intriguingly, *spok* has evolved only in Diptera as a pal- analog of *spook* (*spo*)/*CYP307A* family. In *D. melanogaster*, while *spo* is expressed during early embryonic development and adult ovaries, *spok* is expressed in the PG throughout the larval stages.<sup>19,26</sup> Therefore, duplicated genes are retained but each has acquired a different temporal and tissue-specific expression profile. In accordance with the target, orthologs of *ouib* and *mld* are found only in Diptera so far.<sup>3,19</sup> In contrast, Lepidoptera has only one *spo* gene and has no orthologs of *ouib* or *mld* in the genome. These studies suggest an evolutionary split between Diptera and Lepidoptera in how the ecdysone biosynthesis pathway is regulated during development. Although recently evolved in Diptera lineage, *ouib*, *mld*, and their target *spok* are essential for *D. melanogaster* development. This idea is supported by a report that the newly-evolved, non-conserved genes are indeed essential.<sup>27</sup> Our study reveals a species-specific aspect of ecdysteroid biosynthesis and further studies required to comprehensively understand the molecular mechanisms of steroid hormone biosynthesis in animals.

Are there any other TFs specialized for ecdysteroidogenic enzymes other than Spok? Quite recently, a genome-wide transcriptome analysis and a genome-wide *in vivo* RNAi interference screen were reported and have revealed a number of genes with potential roles in steroidogenesis and developmental timing.<sup>28,29</sup> Since some of the newly identified genes indeed encode TFs, it would be intriguing to examine if these TFs regulate

ecdysteroidogenic enzyme genes in the PG cells. In addition, we are curious to examine if the Drosophilidae genome has any paralogous zinc finger genes to *ouib*. Because the ZAD-Zinc finger genes are strikingly duplicated and diversified in the insect lineage,<sup>30</sup> we hypothesize that any of these zinc finger genes could also be specifically involved in ecdysteroid biosynthesis in the PG.

### Abbreviations

Mld	Molting defective
Ouib	Ouija board
PG	prothoracic gland
Spo	Spook
Spok	Spookier
TF	transcription factor

### Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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