Molecular mechanisms underlying metamorphosis in the most-ancestral winged insect

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Insects comprise over half of the described species, and the acquisition of metamorphosis must have contributed to their diversity and prosperity. The order Odonata (dragonflies and damselflies) is among the most-ancestral insects with drastic morphological changes upon metamorphosis, in which understanding of the molecular mechanisms will provide insight into the evolution of incomplete and complete metamorphosis in insects. In order to identify metamorphosis-related genes in Odonata, we performed comprehensive RNA-sequencing of the blue-tailed damselfly Ischnura senegalensis at different developmental stages. Comparative RNA-sequencing analyses between nymphs and adults identified eight nymph-specific and seven adult-specific transcripts. RNA interference (RNAi) of these candidate genes demonstrated that three transcription factors, Krüppel homolog 1 (Kr-h1), broad, and E93 play important roles in metamorphosis of both Ischnura senegalensis and a phylogenetically distant dragonfly, Pseudethemis zonata. E93 is essential for adult morphogenesis, and RNAi of Kr-h1 induced precocious metamorphosis in epidemis via up-regulation of E93. Precocious metamorphosis was also induced by RNAi of the juvenile hormone receptor Methoprene-tolerant (Med), confirming that the regulation of metamorphosis by the MEKRE93 (Met-Kr-h1-E93) pathway is conserved across diverse insects including the basal insect lineage Odonata. Notably, RNAi of broad produced unique grayish pigmentation on the nymphal abdominal epidemis. Survey of downstream genes for Kr-h1, broad, and E93 uncovered that unlike other insects, broad regulates a substantial number of nymph-specific and adult-specific genes independently of Kr-h1 and E93. These findings highlight the importance of functional changes and rewiring of the transcription factors Kr-h1, broad, and E93 in the evolution of insect metamorphosis.

Insects comprise over half of the described species and are regarded as the most prosperous organismal group in the world (1). Over 98% of the insect species undergo “metamorphosis,” by which they dramatically change their morphology from larvae (nymphs) to adults, with most of them developing wings. Drastic metamorphosis together with the acquisition of wings must have contributed to the remarkable diversity of insects by enabling them to exploit different habitats and resources (2–4).

The genetic and endocrinological mechanisms underlying insect metamorphosis have been elucidated primarily using model insects such as the fruit fly Drosophila melanogaster and the silkworm Bombyx mori, representatives of holometabolous insects that undergo complete metamorphosis with the pupal stage between larva and adult (5). Two insect hormones, juvenile hormone (JH) and ecdysteroid (20-hydroxyecdysone; 20E), regulate insect metamorphosis, and their effects and mechanisms of action have been studied in diverse insects (5, 6). In general, 20E triggers larva (nymph)-to-larva (nymph) ecdysis in the presence of JH, whereas 20E induces pupal and/or adult metamorphosis in the absence of JH. After the elucidation of the intracellular JH receptor gene Methoprene-tolerant (Met) (7, 8), the molecular mechanisms involved in metamorphosis have been functionally analyzed in a variety of insects, including hemimetabolous insects that undergo incomplete metamorphosis from nymphs to adults without the pupal stage.

During larva (nymph)-to-larva (nymph) ecdysis, JH binds to its receptor Met, and subsequently, the transcription factor gene Krüppel homolog 1 (Kr-h1) suppresses metamorphosis to pupa or adult (9). By contrast, during larva (nymph)-to-adult metamorphosis, in the absence of JH, another transcription factor gene E93 induces metamorphosis to adult (10). Kr-h1 inhibits the expression of E93 and vice versa, thereby regulating metamorphosis via the cascade called the MEKRE93 (Met-Kr-h1-E93) pathway (11). The function of MEKRE93 pathway is conserved across holometabolous insects (12, 13) and hemimetabolous insects such as cockroaches (Blattodea) (12), crickets (Orthoptera) (14), and true bugs (Hemiptera) (15, 16). A major difference in the regulatory mechanism of metamorphosis between holometabolous and hemimetabolous insects resides in the contribution of another transcription factor broad (also known as broad-complex) gene. In holometabolous insects, broad triggers

Significance

As caterpillars metamorphose to butterflies, insects change their appearance dramatically through metamorphosis. Some insects have an immobile pupal stage for morphological remodeling (homometabolous). Other insects, such as cockroaches, have no pupal stage, and the juveniles and adults are morphologically similar (hemimetabolous). Notably, among the most-ancestral hemimetabolous insects, dragonflies drastically alter their appearance from aquatic nymphs to aerial adults. In dragonflies, we showed that transcription factors Kr-h1 and E93 are essential for regulating metamorphosis as in other insects, while broad, the master gene for pupation in holometabolous insects, regulates a number of both nymph-specific genes and adult-specific genes, providing insight into what evolutionary trajectory the key transcription factor broad has experienced before ending up with governing pupation and holometabolism.

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pupation. In hemimetabolous insects, meanwhile, broad is primarily involved in wing and ovipositor development (12, 14, 17–19). It should be noted that in the milkweed bug Oncopeltus fasciatus (Hemiptera), in which the function of broad was first analyzed among hemimetabolous insects, RNA interference (RNAi) of broad inhibits the changes in pigmentation patterns that are specific to each nymphal instar, suggesting that broad regulates progressive morphogenesis (18). Three transcription factors, Kr-h1, E93, and broad, have been defined as key factors forming Metamorphic Gene Network (MGN) (20).

The most-ancestral winged insects with metamorphosis are dragonflies and damselflies (Odonata) (Fig. 1 A and B) and mayflies (Ephemeroptera), in which functional analysis of the MGN genes should shed light on the origin of metamorphosis (5, 21, 22). In Odonata, the depletion of JH in final nymphal instar is important for nymph-to-adult metamorphosis, because metamorphosis is suppressed by application of JH analog on the instar is important for nymph-to-adult metamorphosis, because metamorphosis is suppressed by application of JH analog on the first day of final nymphal instar (23), whereas 20E titers increase prior to each ecdysis (24), suggesting that the fluctuation of endogenous JH and 20E titers through postembryonic development in Odonata is similar with that in other hemimetabolous insects (5, 6). However, the molecular mechanisms underlying metamorphosis in Odonata and Ephemeroptera remain largely unknown, mainly due to the lack of molecular genetic tools. We have focused on the blue-tailed damselfly Ischnura senegalensis as a model dragonfly species, established a laboratory-rearing system (25), and developed a method for analyzing gene function by electroporation-mediated RNAi (26, 27).

Here we identified eight nymph-specific and seven adult-specific genes by comprehensive RNA-sequencing of I. senegalensis. Subsequent electroporation-mediated RNAi of the candidate genes and RNA-sequencing after RNAi experiments demonstrated that the interactions of three transcription factor genes, Kr-h1, broad, and E93, were modified through insect evolution for the regulation of metamorphosis.

Results
Screening for Nymph-Specific and Adult-Specific Genes. First, we investigated the gene-expression profiles by comprehensive RNA-sequencing for the following 82 samples from various developmental stages, body regions, and sexes of I. senegalensis: 13 whole-body samples from eggs to seventh instar nymphs (13 developmental stages), 45 samples of penultimate instar and final instar nymphs from head, thorax, abdomen, wing buds, and caudal gills (nine developmental stages), and 24 samples of adults from head, thorax, abdomen, and wings (six individuals consisting of immature and mature males, gynochrome females, and androchrome females) (SI Appendix, Fig. S1 and Table S1).

Here, we adopt the term “nymph” instead of “larva” to distinguish between holometabolous and hemimetabolous insects, although the majority of previous studies on Odonata used the term “larva,” because dragonflies and damselflies drastically change the external morphology through metamorphosis unlike most other hemimetabolous insects (28, 29).

We searched for nymph-specific or adult-specific genes by edgeR analysis (false discovery rate [FDR] < 0.01), in which penultimate instar nymphs and adults were compared for the head, thorax, abdomen, and wings. Here, we used penultimate instar nymphs rather than final instar nymphs (Fig. 1C), considering that the gene-expression patterns may have changed significantly toward adult-like during the final nymphal instar (5). As a result, 64 nymph-specific contigs and eight adult-specific contigs that exhibited differential expression in all the body regions were identified (SI Appendix, Fig. S2). To further screen the candidate genes, we adopted the following three criteria: 1) for one group, minimum TPM (transcripts per million) value is no less than 2; 2) for the other group, maximum TPM value is less than 2; and 3) the minimum TPM value of one group is 1.5 times higher than the maximum TPM value of the other group. Using these criteria, eight nymph-specific contigs and seven adult-specific contigs were detected common to all four body regions (Fig. 1 D and E and Table 1). Using the Integrative Genomics Viewer (30), each contig sequence was carefully examined, and the full-length sequence was manually obtained (Table 1). Of the eight nymph-specific genes (referred to as N1-N8), N4 and N7 corresponded to the transcription factors Kr-h1 and broad, respectively, and of the seven adult-specific genes (referred to as A1-A7), A3 corresponded to the transcription factor E93. The annotation and the expression pattern of each gene are shown in Table 1, Figs. 2A, 3A, and 4A, and SI Appendix, Figs. S3 and S4. Three and eight isoforms were identified for N4 (Kr-h1) and N7 (broad), respectively, while only one isoform was identified for the remaining nymph-specific and adult-specific genes.

Functional Analysis of Nymph-Specific and Adult-Specific Genes. Next, we conducted electroporation-mediated RNAi of the nymph-specific and adult-specific candidate genes. For the nymph-specific genes, double-stranded RNA (dsRNA) was injected at the early stage of the penultimate nymphal instar, and the phenotype was observed after ecdysis to the final instar. For the adult-specific genes, dsRNA was injected at the early...
The RNAi effects of Kr-h1 (Kruppel homolog 1 (D. melanogaster)) in the most-ancestral winged insect indicate molecular mechanisms underlying metamorphosis.

Specific candidate genes, phenotypic effects were detected for one of which (N7 = LC634388-LC634395), 1,641 to 7,876, Transcription factor broad (D. melanogaster) (1 x 10^{-90}).

In several insects, another bHLH-PAS protein called tai (taimannin) forms a complex with Met to bind the JH response DNA motif and induces transcription of target genes (33–35). However, RNAi of tai in I. senegalensis (accession no. LC634405) in penultimate instar nymphs produced wound-like effects on final instar nymphs (10/10 = 100%), and the nymphs died soon after ecdysis (SI Appendix, Fig. S9). The phenotypes of tai RNAi in final instar nymphs changed the color of the adult light-blue regions and inhibited melanin pigmentation (6/10 = 60%) (SI Appendix, Fig. S9).

**Evolution**

**Table 1.** Lists of nymph-specific and adult-specific contigs obtained by the screening criteria in this study.

| Accession no. | Length (bp) | Description (species) | Blastx e-value |
|---------------|-------------|-----------------------|----------------|
| N1            | LC634382    | 1,123                 | Uncharacterized secreted protein |
| N2            | LC634383    | 3,095                 | Peroxidase (D. melanogaster) |
| N3            | LC634384    | 1,205                 | Uncharacterized protein |
| N4            | LC634385    | 6,962                 | Transcription factor Kruppel homolog 1 (D. melanogaster) (1 x 10^{-109}) |
| N5            | LC634386    | 399                   | Uncharacterized secreted protein |
| N6            | LC634387    | 639                   | Uncharacterized secreted protein |
| N7            | LC634388-LC634395 | 1,641 to 7,876        | Transcription factor broad (D. melanogaster) (8 x 10^{-90}) |
| N8            | LC634396    | 896                   | CG64685 transmembrane protein (D. melanogaster) |
| A1            | LC634397    | 1,769                 | Triglyceride lipase (D. melanogaster) |
| A2            | LC634398    | 1,543                 | CG10407 takeout/HBP family protein (D. melanogaster) |
| A3            | LC634399    | 8,790                 | Transcription factor E93 (D. melanogaster) (6 x 10^{-33}) |
| A4            | LC634400    | 1,915                 | Adenylate kinase 3 (D. melanogaster) (1 x 10^{-48}) |
| A5            | LC634401    | 2,512                 | CG31344 ER-bound oxygenase (D. melanogaster) (4 x 10^{-17}) |
| A6            | LC634402    | 1,525                 | Chitinase 10 (D. melanogaster) |
| A7            | LC634403    | 1,850                 | CG9259 Ecdysteroid kinase-like protein (D. melanogaster) (4 x 10^{-26}) |

Bold indicates the genes whose RNAi induced phenotypic effects on the abdominal epidermis of Odonata.

**Kr-h1 and JH Receptor Met Are Essential for Maintaining Nymphal Status in I. senegalensis.** Kr-h1 of I. senegalensis had three isoforms (accession nos. LC634385, LC634606, and LC634607), one of which (Kr-h1a) was expressed until the penultimate nymphal instar and other isoforms were strongly expressed in the late embryonic stage (Fig. 2A and SI Appendix, Fig. S7) as reported in Drosophila (5, 32). RNAi of Kr-h1 (targeting the region common to all isoforms) induced adult-like phenotypes on the nymphal cuticle (16/17 = 94%), of which especially remarkable was the light-blue markings on the eighth abdominal segment of males (white arrowhead; Fig. 2 B and C). Detailed observation on the surface fine structure using scanning electron microscopy (SEM) confirmed that RNAi of Kr-h1 induced adult-like thinner bristles and small granular protrusions on the surface (SI Appendix, Fig. S8A), suggesting that Kr-h1 is essential for maintaining nymphal status in I. senegalensis. When Kr-h1 RNAi was performed in antepenultimate instar nymphs (nymphal instar just before the penultimate instar) nymphs, adult-like phenotypes were also induced (5/8 = 63%) (SI Appendix, Fig. S8 B and C and Table S2).

We also focused on the JH receptor gene to determine whether expression of Kr-h1 in I. senegalensis is JH dependent as in other insects. In insects, the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) protein gene Met is an intracellular receptor for JH (7). The expression of Met in I. senegalensis (accession no. LC634404) was detected at various developmental stages and body regions (Fig. 2A). RNAi of Met in penultimate instar nymphs induced adult-like phenotypes in final instar nymphs after ecdysis (13/14 = 93%), which closely resembled the RNAi phenotypes of Kr-h1 (Fig. 2C and SI Appendix, Fig. S8D). The nymphs with phenotypes induced by RNAi of Kr-h1 or Met failed in ecdysis around the RNAi region during adult emergence (SI Appendix, Fig. S8 E and F). It should be noted that RNAi of Kr-h1 or Met in final instar nymphs did not cause any phenotypic effects in adults (SI Appendix, Fig. SSG).

In several insects, another bHLH-PAS protein called tai (taimannin) forms a complex with Met to bind the JH response DNA motif and induces transcription of target genes (33–35). However, RNAi of tai in I. senegalensis (accession no. LC634405) in penultimate instar nymphs produced wound-like effects on final instar nymphs (10/10 = 100%), and the nymphs died soon after ecdysis (SI Appendix, Fig. S9). The phenotypes of tai RNAi in the red flour beetle Tribolium castaneum (36), the linden bug Pyrrhocoris apterus (37), and the cockroach Blattella germanica (38). In fruit flies and mosquitoes, tai is also important for ecdysteroid-signaling (5, 39), and tai is intimately involved in both JH and ecdysone pathways. The wound-like effects by tai RNAi may be due to an interference with molting in tai RNAi region, because ecdysteroid cannot act properly. RNAi of tai in I. senegalensis in final instar nymphs altered coloration of the adult light-blue regions and inhibited melanin pigmentation (6/10 = 60%) (SI Appendix, Fig. S9).

**E93 is Essential for Adult Morphogenesis in I. senegalensis.** Meanwhile, RNAi of E93 in final instar nymphs changed the color of the adult abdominal epidermis to light brown (13/13 = 100%), which was reminiscent of the nymphal epidermis (Fig. 3). The surface fine structures of the E93 RNAi individuals observed by SEM confirmed that in the E93 RNAi regions (left side in Fig. 3E), bristles were thicker than in the control regions (right side in Fig. 3E), and small granular protrusions on the surface were inconspicuous, closely resembling the nymphal surface structure (Fig. 3 C–G). The nymph-like phenotypes induced by E93 RNAi were also observed in the thorax of adults (SI Appendix, Fig. S10A). When E93 RNAi was performed in penultimate instar nymphs, no phenotypic effect was observed in final instar nymphs (SI Appendix, Fig. S10B), whereas nymph-like epidermal phenotypes appeared in the subsequent emerged adults (SI Appendix, Fig. S10C). Previous studies showed that final instar nymphs of Odonata can be categorized into three developmental stages (stages 1, 2, and 3) based on the morphology of wing sheaths, and the middle stage of I. senegalensis can be further divided into three stages (stage 2a, 2b, and 2c) based on the morphology of compound eyes (31). The phenotypes of E93 RNAi diminished when RNAi was conducted at stage 2 and after (SI Appendix, Fig. S10 D and E), presumably reflecting the increased expression level of E93 at stage 2 and after in the abdomen (Fig. 3A).
RNAi of *broad* Induces Unique Phenotypes in *I. senegalensis*. As in other insects, the *broad* gene transcripts consisted of multiple isoforms with different 3′ end (*SI Appendix*, Fig. S11). The protein encoded by *broad* gene contains a core region consisting of a Broad-Complex-Tramtrack-Bric-a-brac (BTB) domain and a linker region that are commonly found among the isoforms and a C2H2 zinc-finger domain that is alternatively spliced. The C2H2 zinc-finger domains in the *broad* gene transcripts are classified into six clades (5, 40, 41). We identified seven zinc-finger domains from *broad* gene transcripts of *I. senegalensis* (accession nos. LC634388 through LC634395) (*SI Appendix*, Fig. S11), which were classified into five clades except for the cockroach-specific ZF6 clade (Fig. 4). When we also examined the published genomes of two Odonata species, *Ladona* (= *Libellula*) fulva (Anisoptera; Libellulidae; GenBank: GCA_000376725.2) (42) and *Ischnura elegans* (Zygoptera; Coenagrionidae) (43), seven zinc-finger domains were identified in both species (Fig. 4B and *SI Appendix*, Fig. S11A). Therefore, we named seven zinc-finger domains conserved in Odonata as ZFa–ZFg in order from the 5′ side (*SI Appendix*, Fig. S11A). In *I. senegalensis*, the expression patterns of the isoform were similar to each other, among which ZFd and ZFe exhibited relatively high expression levels (*SI Appendix*, Fig. S11B). All isoforms were outstandingly expressed in caudal gills, a nymph-specific organ (Fig. 1C, arrowheads), during the final nymphal instar (Fig. 4A and *SI Appendix*, Fig. S11B). Since the caudal gills are spontaneously excised by stimulation like electroporation, the effect of RNAi could not be examined.

RNAi targeting the *broad* common region (BTB domain or linker region) induced a grayish pigmentation in the nymphal epidermis of both males and females (white arrowheads, Fig. 4C and D), which was distinct from the phenotypes of *Kr-h1* RNAi. The surface fine structures exhibited no obvious differences between the *broad* RNAi regions and the control regions (*SI Appendix*, Fig. S12). Like *Kr-h1* RNAi, the nymphs with phenotypes induced by *broad* RNAi failed in ecdysis around the RNAi region upon adult emergence (*SI Appendix*, Fig. S12D). It should be noted that *broad* RNAi in final instar nymphs caused no phenotypic effects in adults (*SI Appendix*, Fig. S12E). Single RNAi experiments specifically targeting either ZFd, ZFe, or ZFf exon were performed to identify the function of each isoform, but none of them affected the epidermal pigmentation. On the other hand, double RNAi of ZFd and ZFe and multiple RNAi of seven isoforms induced the grayish epidermal phenotype as...
observed in RNAi targeting the common region (Fig. 4C), suggesting that these isoforms have redundant function in the epidermal pigmentation. Because broad is known to be involved in wing development in Hemiptera (18, 44), Orthoptera (14), and Blattodea (19), we attempted to perform RNAi experiments against wings of Odontotettix. However, when electroporation was applied at 25 V, the same voltage as that applied to the epidermis, wing formation was inhibited even in the negative control (SI Appendix, Fig. S13A). When the voltage was lowered to 10 V, the detrimental effects in the negative control were attenuated, but the phenotypic effects of Kr-h1 RNAi and broad RNAi were not observed (SI Appendix, Fig. S13B). These results suggested that the electroporation-mediated RNAi is not applicable to the wings of penultimate instar nymphs of I. senegalensis.

Gene-Expression Profiles Associated with RNAi of Kr-h1, broad, and E93. In I. senegalensis, as in other insects, it was confirmed that Kr-h1 RNAi induced precocious adult phenotypes and E93 RNAi retained nymphal phenotypes. Meanwhile, the morphological phenotypes of broad RNAi were difficult to interpret. Therefore, gene-expression patterns of the abdominal epidermis caused by RNAi of Kr-h1 (n = 3), broad (n = 4), and E93 (n = 3) were analyzed by RNA-sequencing. For comparison, samples of the abdominal epidermis from untreated final instar nymphs (1 d after nymphal ecdysis) and adults (1 to 2 d after adult emergence) were also used. We identified 166 nymphal-epidermis-specific (NES) transcripts (158 genes plus eight isoforms) and 610 adult-epidermis-specific (AES) transcripts (595 genes plus 15 isoforms) from the untreated final instar nymphs (n = 12) and adults (n = 10) based on the following criteria: 1) edgeR analysis (FDR < 0.01), and 2) the minimum TPM value of one group is more than 1.5 times higher than the maximum TPM value of the other group (Dataset S1). By focusing on the NES and AES genes, we constructed hierarchical cluster dendrograms, which confirmed that the gene-expression patterns of the Kr-h1 RNAi regions became close to those of untreated adults (Fig. 5A). Notably, some of the AES genes were strongly up-regulated in the broad RNAi regions, uncovering that the gene-expression patterns of the broad RNAi regions were partially similar to those in adults (Fig. 5A, asterisk). By contrast, the gene-expression patterns of the E93 RNAi regions were globally similar to those of final instar nymphs (Fig. 5B).

Screening of Candidate Downstream Genes for Kr-h1, broad, and E93. We confirmed that broad RNAi certainly reduced the expression levels of broad and so did E93 RNAi the expression levels of E93 (Fig. 5 C and D and SI Appendix, Fig. S14A and
As reported in lepidopteran species, since the RNAi regions exhibit mosaicism, the samples from the RNAi regions contain both affected and unaffected cells, so the reduction of the expression levels cannot be high inherently. On the grounds that the RNAi region and the control region were compared within the same individual, we tested the statistical significance of the difference by paired t-test. We examined how these three transcription factors interact with each other. RNAi of Kr-h1 reduced the expression of broad and increased the expression of E93 (Fig. 5C and D and SI Appendix, Fig. S14A and B), as reported in other hemimetabolous insects. RNAi of broad affected neither the expression of Kr-h1 nor the expression of E93 (Fig. 5D and E and SI Appendix, Fig. S14B and C). RNAi of E93 increased the expression of broad but did not affect the expression of Kr-h1 (Fig. 5C and E and SI Appendix, Fig. S14A and C). While the expression levels of Kr-h1 were consistently very low in final instar nymphs, the expression levels of Kr-h1 in penultimate instar nymphs were substantial and reduced by Kr-h1 RNAi (Fig. 5E and F and SI Appendix, Fig. S14C).

Next, we surveyed target genes for the three transcription factors Kr-h1, broad, and E93 (Dataset S1). More than 90% of both the NES and AES genes were consistently affected in all individuals by RNAi of at least one of the three transcription factors Kr-h1, broad, and E93.
Fig. 5. Summary of RNA-sequencing in Kr-h1 (n = 3), broad (n = 4), and E93 (n = 3) RNAi individuals. (A and B) Clustering dendrogram and heatmap on the differential gene expression of 166 NES and 610 AES transcripts. Magenta color is in high expression level, while green color is in low expression level. Color key value indicates the log2 fold change in the expression levels. Asterisk indicates genes whose expressions were induced by both Kr-h1 RNAi and broad RNAi individuals. (A) Kr-h1 (n = 3) and broad (n = 4) RNAi individuals. (B) E93 (n = 3) RNAi individuals. (C–E) Expression level changes of broad (sum of all isoforms) (C), E93 (D), and Kr-h1 (E) genes after RNAi treatments. (F) Expression level changes of Kr-h1 between Kr-h1 RNAi region and control region in the penultimate instar. (G and H) Venn diagrams of 166 NES (G) and 610 AES (H) transcripts. Each circle indicates genes satisfying these criteria: 1) the average expression in the RNAi regions exhibits a more-than-1.5-fold change compared with the average expression in the control regions, and 2) all individuals showed the same tendency for increase or decrease. P value between untreated nymphs and adults was calculated by Student's t test, while P value between RNAi regions and control regions was calculated by paired t test. Error bars are SD.
factors, while exhibiting a more-than-1.5-fold change in the average expression level in the RNAi regions compared with the control regions (Fig. 5 G and H). For example, the expression of 24 NES genes (Fig. 5G; as an example, NES013: Cuticular protein CPR1-type CPR-L5 is shown in SI Appendix, Fig. S14D) increased more than 1.5-fold by the RNAi of Kr-h1 but decreased to less than 2/3 by Kr-h1 and broad RNAi, whereas the expression of 128 AES transcripts (125 genes plus three iso-forms) (Fig. 5H; as an example, AES021: Cuticular protein CPCFC-A2 is shown in SI Appendix, Fig. S14E) increased more than 1.5-fold by the RNAi of Kr-h1 and broad but decreased to less than 2/3 by the RNAi of E93. Since the expression of Kr-h1 and E93 is not affected by broad RNAi, these results indicate that broad regulates a substantial number of NES (24 + 27 + 6 + 11 transcripts; 68/166) and AES (128 + 100 + 7 + 3 transcripts; 238/610) genes without being mediated by Kr-h1 or E93. In other words, a number of NES genes (9 + 47 + 30 transcripts; 86/166) (Fig. 5G; as an example, NES038: Cuticular protein CPLCP-L1 is shown in SI Appendix, Fig. S14F) and AES genes (129 + 153 + 57 transcripts; 339/610) (Fig. 5H; as an example, AES050: 1-Acetylglcerol-3-phosphate O-acyltransferase 2 is shown in SI Appendix, Fig. S14G and Dataset S1) were affected by RNAi of E93 and/or Kr-h1 but not by RNAi of broad, suggesting that E93 and Kr-h1 regulate the expression of several genes without involvement of broad. It should be noted that NES genes (9 + 27 transcripts) and AES genes (129 + 100 transcripts) were affected by RNAi of Kr-h1 but not by RNAi of E93 in our criteria (Fig. 5 G and H). While some of these genes are likely to be near the border of the criteria, at least some of them showed marked effects by RNAi of Kr-h1 but little or no effects by RNAi of E93 (as an example, NES038: Cuticular protein CPLCP-L1 is shown in SI Appendix, Fig. S14F and Dataset S1), suggesting that Kr-h1 may partly regulate the nymphal status independently of E93. Although 11 NES transcripts (NES055a, NES055b, NES058, NES095, NES097, NES115, NES119, NES121, NES143, and NES152) and three AES genes (AES135, AES162, and AES572) were only affected by RNAi of broad in our criteria (Fig. 5 G and H), similar expression change was observed by RNAi of Kr-h1 in all these genes (but they did not meet the criteria) (Dataset S1). Since broad is induced by Kr-h1 (Fig. 5C), these genes are likely to be controlled by both Kr-h1 and broad.

Gene ontology analyses revealed that NES genes contained many structural molecule proteins (e.g., cuticular proteins), whereas AES genes contained many enzymes, binding proteins, transporters, and structural molecule proteins (SI Appendix, Fig. S15A). We confirmed that a variety of genes were affected by RNAi of broad (SI Appendix, Fig. S15A and Dataset S1). Among NES and AES genes, we identified 47 and 26 cuticular proteins, respectively (SI Appendix, Fig. S15B and Dataset S1). Based on the characteristic motif classification (46), cuticular protein genes with R11 motif were predominantly found in NES genes, while cuticular protein genes with CPCFC motif were identified only from AES genes (SI Appendix, Fig. S15B). Interestingly, most of AES cuticular protein genes were down-regulated by broad (SI Appendix, Fig. S15B and Dataset S1). Nymphal and adult-specific cuticular genes seem to be involved in the formation of their characteristic surface structures (Fig. 3 C-G and SI Appendix, Fig. S8 A and D). Moreover, 16 genes involved in pigmentation (four genes for melanin, one gene for ommochrome, three genes for ommochrome, pteridine, and uric acid, and eight genes for pteridine and uric acid) were identified from AES genes (SI Appendix, Fig. S15C and Dataset S1). Because light-blue coloration in adult males is produced by coherent light-scattering due to the light-scattering granules containing pteridine pigments and the light-absorbing granules containing ommochrome pigments and black coloration in adults is produced by melanin pigments (47, 48), these genes may be involved in the formation of colorful adult pigmentation. Furthermore, among NES or AES genes, 12 transcription factors (e.g., Blimp-1 and HR4) and 19 signaling receptor-related proteins (e.g., hedgehog and EGF-R) were identified (SI Appendix, Figs. S15 D and E and Dataset S1). The biological role of these genes in metamorphosis deserves further study.

**Gene-Expression and Functional Analysis in Pied Skimmer Dragonfly* Pseudomethis zonata.** The four genes, Kr-h1, Met, E93, and broad, which were shown to affect metamorphosis in the blue-tailed damselfly *I. senegalensis*, were also analyzed in a phylogenetically distant dragonfly *Pseudomethis zonata* (Anisoptera; Libellulidae) (accession nos. LC634406–LC634416). As in *I. senegalensis*, eight isoforms of broad (Fig. 4B and SI Appendix, Fig. S16) (accession nos. LC634409–LC634416) and two isoforms of Kr-h1 were identified in *P. zonata* (SI Appendix, Fig. S7) (accession nos. LC634406 and LC636406). The expression patterns of these genes in *P. zonata* were similar to those in *I. senegalensis*, except that Kr-h1 was highly expressed in the abdomen of females even after the final nymphal instar (Fig. 6 A–D and SI Appendix, Fig. S7). Kr-h1 expression in female final instar nymphs could be related to JH-dependent reproductive activities, such as the production of yolk proteins (5).

Adult-specific yellow markings were induced by the RNAi of Kr-h1 (28/31 = 88%) or Met (29/33 = 88%) when RNAi was conducted in the penultimate or antepenultimate instar nymphs (Fig. 6 E–J and SI Appendix, Fig. S17 and Table S2). The effect of broad RNAi was subtle, only slightly increasing blackness in several individuals (4/30 = 13%) (Fig. 6 K and L and SI Appendix, Fig. S17 and Table S2). By contrast, E93 RNAi in final instar nymphs produced nymph-like coloration and surface structures (Fig. 6 M–Q; nymph-specific bristles are indicated by gray arrowheads) in adults (8/8 = 100%), as observed in *I. senegalensis*.

**Discussion**

The Functional Role of the MEKRE93 Pathway in Metamorphosis of *Odonata.* Comprehensive gene-expression analysis and RNAi functional analysis confirmed that three transcription factors, Kr-h1, broad, and E93, are essential for metamorphosis in Odonata, at least in the abdominal epidermis. Although dragonflies and damselflies greatly change their morphology from nymphs (often called larvae) to adults, the presence of broad expression from embryogenesis until the final nymphal instar is consistent with the cases of other hemimetabolous insects. Among hemimetabolous insects, such a large-scale transcriptomic analysis during metamorphosis has been reported only for cockroaches (49), and the comprehensive screening of metamorphosis-related genes with gene functional analysis as reported in this study is unique.

In some insect species such as the fruit fly *D. melanogaster*, adult epidermal cells proliferate from the histoblast nests, while in other insect species such as the tobacco hornworm *Manduca sexta*, the larval epidermal cells persist through metamorphosis and synthesize larval, pupal, and adult cuticles continuously (5, 50). In Odonata, the RNAi phenotype by electroporation shows finely mosaic patterns and varies between individuals, suggesting that the development of adult epidermal cells is likely to be derived from larval epidermal cells.

In other insects, Met and Kr-h1 RNAi were reported to cause precocious metamorphosis (7–9, 44, 51–53), and E93 RNAi showed retention of larval/nymphal morphology and larva-/nymph-specific gene-expression pattern (12, 14). The rewiring of the relationship of genes involved in metamorphosis during insect evolution is shown in Fig. 7. Comparison of gene-expression patterns in RNAi individuals showed that Kr-h1 suppresses E93 (Figs. 5 and 7 and SI Appendix, Fig. S18), as
reported in other insects (12–16), indicating that the central part of the MEKRE93 pathway is conserved in Odonata. In the mayfly *Cloeon viridulum*, both *Kr-h1* and *broad* were highly expressed at the nymphal stage (54). Although functional analysis of these genes and expression of *E93* have not been reported from Ephemeroptera, these results are concordant with our results in Odonata.

Unlike other insect groups (5, 12, 14), suppression of *Kr-h1* expression by *E93* was not observed in this study: the expression of *Kr-h1* did not increase in the RNAi regions of *E93*, at least in the abdominal epidermis during the final nymphal instar (Figs. 5 and 7 and SI Appendix, Fig. S14), despite the retainment of nymphal morphology (Figs. 3 and 6). This result can be interpreted in two ways. First, dragonflies and damselflies are different from other insect groups. Alternatively, because previous studies used systemic RNAi, the suppression of the gene expression in one tissue can be affected by other tissues indirectly. For example, an increase in *E93* in the corpora allata (JH-producing organ) at the onset of metamorphosis may be involved in the cessation of JH synthesis, and systemic

Fig. 6. Molecular mechanisms underlying metamorphosis in the pied skimmer dragonfly *P. zonata*. (A–D) Expression levels. (A) *Kr-h1*, (B) *Met*, (C) *E93*, and (D) *broad*. M, F, AP, and P indicate male, female, antepenultimate instar nymphs, and penultimate instar nymphs, respectively. (E) A final instar nymph. (F) Adult male with yellow coloration on the third and fourth abdominal segments. (G–L) RNAi phenotypes in the final instar nymphs. (G and H) *Kr-h1*, (I and J) *Met*, and (K and L) *broad*. (H, I, and L) show magnified views of G, I, and K, respectively. White and black arrowheads indicate adult-specific yellow coloration and grayish pigmentation, respectively. (M) Ventral RNAi phenotypes of negative control (*bla RNAi*). (N) Ventral RNAi phenotypes of *E93*. (O–Q) SEM observation on the abdominal surface of *E93* RNAi individuals. (O) An adult without any treatments. (P) *E93* RNAi individual. *E93* RNAi region is on the right side of the photo. (Q) A final instar nymph without any treatments. Gray arrowheads indicate nymph-specific bristles on the abdominal surface.
E93 RNAi could affect the corpora allata, allowing production of JH, thereby increasing Kr-h1 expression. In each body part (head, thorax, abdomen, and wings), the expression of E93 was almost undetectable in the penultimate nymphal instar, detected at early stage 1 of the final nymphal instar, and drastically up-regulated at stage 2 of the final nymphal instar and on (Figs. 3A and 6C). The effect of E93 RNAi was confirmed up to early stage 2 of the final nymphal instar (SI Appendix, Fig. S10E), which was more than 2 wk after the depletion of Kr-h1 expression (Fig. 2A), suggesting that the loss of Kr-h1 expression is not attributable to E93 expression.

Function of broad Gene in Odonata and Evolution of Metamorphosis in Insects. The most-noteworthy finding in this study is the role of broad gene in Odonata. In holometabolous insects, broad is specifically expressed in the pupal development and is essential for pupation (55–58). Meanwhile, in hemimetabolous insects without pupal stage, broad gene is highly expressed during the nymphal stage and is mainly involved in the development of adult wings and ovipositor (5, 12, 14, 18, 44). It should be noted that broad gene has also been identified in ametabolous firebrat Thermobia domestica (Zygentoma), although its function has not been examined (59). In Odonata, broad was exclusively expressed during the nymphal period, and RNAi of broad induced adult-like pigmentation (Figs. 4C and 6K and L) but did not significantly change the nymphal surface structures (SI Appendix, Fig. S12). It should be noted that some change in pigmentation patterns by RNAi of broad has also been reported from the milkweed bug (18).

In the broad RNAi nymphs, expression levels of both Kr-h1 and E93 were unchanged, while expression levels of many of NES and AES genes were affected (Fig. 5). These results strongly suggest that broad gene regulates these genes without being mediated by Kr-h1 and E93 (Fig. 7 and SI Appendix, Fig. S18). The contribution of broad to the repression of genes involved in adult status is similar to that in holometabolous insects (5), while the contribution of broad to the induction of genes involved in nymphal status is, to our knowledge, previously undescribed (Figs. 5 and 7 and SI Appendix, Fig. S18). The contribution of broad to the repression of genes involved in adult status is similar to that in holometabolous insects (5), while the contribution of broad to the induction of genes involved in nymphal status is, to our knowledge, previously undescribed (Figs. 5 and 7 and SI Appendix, Fig. S18). Our results suggest that broad is a major player downstream of Kr-h1 in maintaining juvenile traits and suppressing adult traits in nymphs of Odonata. In adults of Odonata, E93 represses the broad gene as in other insects (Figs. 5 and 7 and SI Appendix, Figs. S14 and S18) (5).
The RNAi phenotypes of broad were different from those of Kr-h1 (Figs. 2 and 4 and SI Appendix, Figs. S8 and S11), which can be explained as follows. Because RNAi of broad does not induce E93 expression (Fig. 5 and SI Appendix, Fig. S14B), the adult status controlled by E93 cannot be reproduced (Fig. 7). In addition, since broad regulates a subset of nymph-specific and hemimetabolous genes without being regulated by E93 or Kr-h1 (Figs. 5 and 7), RNAi of broad may end up with unique phenotypes that are distinct from both nymphs and adults (Fig. 4).

It has been argued that functional differences in broad gene across different insect groups are important in understanding the evolution of metamorphosis (5, 60, 61). Previously, broad gene was shown to be mostly involved in the development of wings and ovipositor in hemimetabolous insects based on the RNAi phenotypes, but its role in other tissues has been unknown. In this study, we demonstrate that in Odonata species whose body coloration differs between adults and nymphs, the broad gene is involved in the development of the abdominal epidermis, at least to some extent. We also suggest that plausibly, the effects of broad RNAi may be different across insect groups, on the grounds that broad suppresses E93 in other hemimetabolous insects (Fig. 7). In holometabolous insects, another major change must have occurred: Kr-h1 does not induce but suppresses E93 (5). Modification and rewiring of the relationships across the transcription factors Kr-h1, broad, and E93 may have contributed to the evolution of insect metamorphosis, and therefore, it is necessary to clarify when and how these changes have occurred in the evolution of diverse insects. In this study, we also identified an array of genes potentially regulated by broad gene in Odonata (Fig. 5 G and H and SI Appendix, Figs. S14–D and S15 and Dataset S1). Investigation of expression, regulation, and functions of these genes in comparison with other insect systems would provide insight into the evolutionary origin of insect pupa and holometabolism.

Materials and Methods
Complete details on the materials and methods are available in SI Appendix.

Nymphs and adults of I. senegalensis and P. zonata used in this study were collected in Tsukuba, Ibaraki, Japan. Some eggs of I. senegalensis were obtained from wild mature females and reared in the laboratory as described (25). Total RNA was extracted from the freshly prepared samples using RNeasy Plus (Takara Bio), and complementary DNA libraries were constructed using TruSeq RNA Sample Preparation Kits version 2 (Illumina) and sequenced by HiSeq (Illumina). The sample information and RNA-sequencing reads are shown in SI Appendix, Table S1. Transcriptome analyses were performed as described (62). Electroporation-mediated RNAi was conducted as described previously (26, 27). As a negative control, we used a dsRNA targeting β-lactamase (bla) gene. The primer sets to produce templates for dsRNA synthesis are shown in SI Appendix, Table S3. RNAi targeting the multiple genes was performed using an equivalent mixture of 100 μM dsRNA solutions (i.e., final concentration was 50 μM each for the RNAi of two genes). The overall results of RNAi experiments were summarized in SI Appendix, Table S2.

Data Availability. DNA sequences and raw Fastq data have been deposited in DNA Data Bank Japan Read Archive (LC633482–LC634416, LC634606–LC634608, DRR278508–DRR278641, and DRR300699–DRR300702). All other study data are included in the article and/or supporting information.

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