A feminizing switch in a hemimetabolous insect

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The mechanism of sex determination remains poorly understood in hemimetabolous insects. Here, in the brown planthopper (BPH), Nilaparvata lugens, a hemipteran rice pest, we identified a feminizing switch or a female determinant (Nlfmd) that encodes a serine/arginine-rich protein. Knockdown of Nlfmd in female nymphs resulted in masculinization of both the somatic morphology and doublesex splicing. The female-specific isoform of Nlfmd, Nlfmd-F, is maternally deposited and zygotically transcribed. Depletion of Nlfmd by maternal RNAi or CRISPR-Cas9 resulted in female-specific embryonic lethality. Knockdown of an hnRNP40 family gene named female determinant 2 (Nlfmd2) also conferred masculinization. In vitro experiments that showed an Nlfmd2 isoform, Nlfmd2\(^{340}\), bound the RAAGAA repeat motif in the Nldsx pre-mRNA and formed a protein complex with NIFMD-F to modulate Nldsx splicing, suggesting that NIFMD2 may function as an RNA binding partner of the feminizing switch NIFMD. Our results provide novel insights into the diverse mechanisms of insect sex determination.

INTRODUCTION

Insects have evolved an astonishing variety of molecular mechanisms to achieve sex determination (1). Although doublesex (dsx) is the conserved gene at the bottom of the sex determination cascade, the primary signals are diverse among different insect species (2). In the fruit fly Drosophila melanogaster, the dosage of four X-encoded signal element (XSE) proteins determines sex (3). In housefly Musca domestica, the male sex is determined by Mdmd, which is a paralog of the generic splice factor gene CW22 (4). In the medfly Ceratitis capitata, Maleness-on-the-Y (MoY) orchestrates male sex determination (5). In the yellow fever mosquito Aedes aegypti, an RNA binding protein, NIX, serves as the primary male-determining signal (6). In the rice planthopper (BPH), Laodelphax striatellus (female, XX; male, XO) and Sogotalla furcifera (female, XX; male, X0) display important roles of effective RNA interference (RNAi) and parental RNAi. Furthermore, the Ccdsx and Ccsxl orthologs exist in three hemimetabolous insects (7–10).

In the sex determination cascade, proteins that directly regulate the alternative RNA splicing of dsx have been identified only in a few insect species belonging to different orders. In the dipteran species D. melanogaster, transformer (Dmtra) is regulated by sex-lethal (Dmtra2) and displays sex-specific splicing isoform. The female-specific isoform Dmtra forms a complex with transformer2 (Dmtra2) to regulate the alternative RNA splicing of dsx pre-mRNA (11–13). The DmTRA protein belongs to the serine-arginine (SR) protein family but lacks an RNA recognition motif (RRM), which is essential for binding of pre-mRNA, whereas DmTRA2 in the complex provides the RRM domain. In females, DmTRA/DmTRA2 binds the exon splicing enhancer (ESE) (TC(T/A)(T/A)CAATCAACA), in exon 4 of the dsx pre-mRNA, stimulating the use of a weak 3′ splice site to retain exon 4 (14–16). In male D. melanogaster, the male-specific Dmdsx is produced by default as the weak 3′ splice site is not used in the absence of the female-specific TRA/TRA2 complex, skipping the female-specific exon 4. In another dipteran species, C. capitata, TRA and TRA2 orthologs, CcTRA and CcTRA2, regulate the splicing of the Ccdsx pre-mRNA. However, Ccsxl is not involved in sex determination, and Cctra acts as cellular memory maintaining the female pathway by autoregulation with the help of Cctra2 (17–20). TRA orthologs appear to be absent in mosquitoes, which also belong to the order Diptera (21). In Hymenoptera species, A. mellifera, the gene Amfem encodes an SR-type protein. In addition, fem functions similarly to Cctra in sex determination with regulation of doublesex (Amdsx) and autoregulation (22–24). Moreover, no TRA homolog has been identified in B. mori, and Bmtra2 is not involved in the regulation of sex-specific Bmdsx pre-mRNA splicing (25, 26).

Knowledge of the mechanism of sex determination in hemimetabolous insects is still limited. The Hemiptera brown planthopper (BPH), Nilaparvata lugens, is the most destructive rice pest in Asia (27). BPH is also an excellent model system to study the sex determination pathway of hemimetabolous insects with the availability of effective RNA interference (RNAi) and parental RNAi. Furthermore, the CRISPR-Cas9 system for BPH has also been successfully established as reported recently (28, 29). Recent studies have indicated that BPH uses the XX (female) and XY (male) system for sex determination, which is different from other known hemipteran species, such as Sogotalla furcifera (female, XX; male, XO) and Laodelphax striatellus (female, XX; male, XO) (30). We previously showed that sex-specific splicing of Nldsx plays important roles during sexual differentiation in BPH. Nldsx controlled sexual dimorphism based on male-specific expression; males were strongly feminized following Nldsx RNAi knockdown, while females developed normally (31). In our previous study, the xel homolog Nlxel was not involved in sex determination in BPH. Recently, Wexler et al. reported that a tra ortholog existed in three hemimetabolous insects including a Hemiptera species, Rhodnius prolirius (32). In this study, we sequenced the early embryonic transcriptome and performed a large-scale screen using RNAi to identify the upstream regulators of Nldsx. We identified two genes involved in the sex determination cascade of BPH, tentatively named Female determinant factor 1 (Nlfmd) and Female determinant factor 2 (Nlfmd2), respectively.
We present evidence suggesting that \textit{Nlfmd-F}, the female-specific isoform of \textit{Nlfm}, is a feminizing switch that modulates sex-specific \textit{Nldxs} splicing. Our work yielded new insight into the RNA binding protein partner and the ESE associated with the feminizing switch. \textit{Nlfmd} homologs that showed similar functions were identified from two other hemipteran species, \textit{S. furcifera} and \textit{L. striatellus}, suggesting a conserved role in several hemipteran species.

**RESULTS**

\textit{Nlfmd} encodes a Ser/Arg-rich (SR-rich) protein required for female development and sex-specific alternative splicing of \textit{Nldxs}

To discover candidate genes that are involved in sex determination, we performed a large-scale screen targeting more than 200 \textit{N. lugens} embryonic transcripts (supplementary file S1) by injecting their corresponding double-stranded RNAs (dsRNAs) in the third instar nympha of \textit{N. lugens}. A gene \textit{Nlfmd}, which encodes an SR-type protein, was found from this screen as its knockdown resulted in partial masculinization of females, exhibiting shortened ovipositor and appearance of male external genitalia. According to reverse-transcription polymerase chain reaction (RT-PCR), \textit{Nlfmd} has two isoforms, a female-specific \textit{Nlfmd-F} (GenBank accession number MW082042) and a non-sex-specific \textit{Nlfmd-C} (GenBank accession number MW082041) (Fig. 1A). \textit{Nlfmd-F} skips a 150–base pair (bp) exon 6 and a part of the exon 13, resulting in the loss of the 50–amino acid residues encoded by exon 6 and an alternative stop codon in the female-specific exon 14. \textit{Nlfmd-F} encodes a 613–amino acid protein that contains an Arg/Ser-rich domain, a putative autoregulatory domain, and a Pro-rich region. Although a protein BLAST (basic local alignment search tool) search in the National Center for Biotechnology Information (NCBI) failed to identify any close homologs other than what is reported in this study, the putative autoregulatory domain of \textit{Nlfmd} showed some similarity to the hymenopteran protein \textit{A. mellifera} feminizer (AmFem) (brown box in Fig. 1B). However, the overall protein sequence identity between \textit{Nlfmd-F} and AmFem, AmCSD, DmTRA, and CcTRA is 5.34, 8.09, 5.50, and 6.63%, respectively, as measured by DNAman (www.lynnon.com/). Moreover, the order of the three domains are not conserved between \textit{Nlfmd} and these TRA/FEM family proteins (33).

Having characterized the isoforms of \textit{Nlfmd}, we performed further RNAi experiments using a dsRNA that targets the common region (exons 1, 2, 3, and 4) of the two \textit{Nlfmd} transcripts (fig. S1A). The results of the RNAi showed that all females with RNAi knockdown of \textit{Nlfmd} in the third instar developed into intersexes, showing phenotypes with smaller body, shorter ovipositor, male-specific genital segment, and undeveloped ovaries, indicating that the influence of \textit{Nlfmd} was systemic (Fig. 1, D and E, figs. S2 and S3, and table S1). However, knockdown of \textit{Nlfmd} in the males did not cause any obvious somatic phenotype or loss of fertility when compared with the control groups (Fig. 1D, fig. S2, and Table 1). Injection of dsRNA targeting the \textit{Nlfmd-F}–specific exon 14 into the third instar nymphs resulted in the same phenotypes compared to the knockdown of the common regions of \textit{Nlfmd-F} and \textit{Nlfmd-C} (fig. S4).

To investigate the impact of \textit{Nlfmd} on the sex-specific splicing of \textit{Nldxs}, we quantified the sex-specific \textit{Nldxs} transcripts in BPH adults following injection of \textit{dsgfp}, \textit{dsNlfmd} to the third instar nymph stage, respectively. Although having no notable effect in males, the knockdown of \textit{Nlfmd} in females significantly decreased the relative level of the female-specific \textit{Nldxs-F} and increased the relative level of the male-specific \textit{Nldxs-M}, compared to the \textit{dsgfp} controls (Fig. 1, F and G). Thus, \textit{Nlfmd} is upstream of \textit{Nldxs} in the sex determination pathway of BPH.

To investigate whether \textit{fmd} is conserved in other members of Hemiptera, we identified homologous genes in two other hemipteran insects from \textit{S. furcifera} (\textit{Sffmd}) and \textit{L. striatellus} (\textit{Lsfmd}), respectively. Knockdown of either \textit{Sffmd} or \textit{Lsfmd} resulted in females developing into intersexes similar to \textit{Nlfmd} (figs. S6 and S7). These results suggest that the role of \textit{fmd} in female development is conserved in hemipteran insects in at least three different genera.

\textit{Nlfmd-F} is transcribed before the appearance of \textit{Nldxs-F} in early embryos, and \textit{Nlfmd} depletion results in female-specific embryonic lethality

Transcripts of \textit{Nlfmd} could be detected by RT-PCR in all developmental stages starting from newly oviposited eggs, indicating a maternal contribution. However, the expression of \textit{Nlfmd-F} decreased sharply in the 12th-hour embryos, and then increased in the 24th-hour embryos (Fig. 2, A and B). We also detected the expression of \textit{Nlfmd} transcripts in individual eggs at different embryonic development stages, and the results showed that zygotic \textit{Nlfmd-F} was established between 12 and 24 hours before the initial detection of the female-specific \textit{Nldxs-F} between 48 and 60 hours after oviposition (fig. S8). In addition, \textit{Nlfmd-F} transcripts can be detected in all tissues tested in adult female BPHs (fig. S5A).

Knockdown of \textit{Nlfmd} by maternal RNAi in newly emerged females reduced the number of eggs to only 10% of that of the \textit{dsgfp}-treated controls (Fig. 2C and fig. S9A). Moreover, of these eggs, only approximately 50% hatched, and they were all XY males (Fig. 2, C and D). Loss of \textit{Nlfmd} activity appears lethal to the female embryos but has no obvious impact to the male embryos. There was no detectable difference in the fertility of these male offspring compared to that of the control males (table S2).

CRISPR-Cas9 was also used to knock out \textit{Nlfmd}. Only heterozygous male mutants were obtained in \textit{G0}, according to genotype analysis performed using adult wings (Fig. 3, A and B). No homozygous female mutants were found in the \textit{G1} progeny from a cross between heterozygous \textit{G1} mutant males and females (Fig. 3B-b3). However, a number of eggs that showed embryonic arrest were found in \textit{G2}, and genotyping analysis showed that they were XX females. This is consistent with the earlier maternal RNAi results suggesting that either maternal or early zygotic \textit{Nlfmd} is required for female embryonic development (Fig. 2C). Homozygous \textit{G2} mutant males were found, but the number was less than expected, suggesting that \textit{Nlfmd}, presumably the non–sex-specific \textit{Nlfmd-C}, may be involved in male development (Fig. 3, C and D).

\textit{Nlfmd2} also affects female development and \textit{Nldxs} splicing

During our RNAi screen, we also found a second gene that is involved in sex determination in BPH, and we named it \textit{Female determiner 2} (\textit{Nlfmd2}). \textit{Nlfmd2} encodes multiple protein isoforms with two RRMs. They belong to a conserved and widely distributed RNA binding protein family that includes the \textit{D. melanogaster} SQUID protein and other heterogeneous nuclear ribonucleoprotein 40 proteins (hnRNP40), but not the TRA2 family of proteins (fig. S10). Gene \textit{Nlfmd2} had four alternative splicing isoforms in both sexes and potentially encode three different proteins (\textit{Nlfmd2}^340, \textit{Nlfmd2}^387, and}
Nlfmd262; GenBank accession numbers MW082037, MW082038, and MW082039/MW082040) (Fig. 4, A and C). In the four transcripts, exon2 and exon3 were retained in Nlfmd262, but spliced out in Nlfmd2340 and Nlfmd2338, and exon 2 contained a stop codon that prematurely terminated the translation of Nlfmd262 (Fig. 4A). Nlfmd2340 and Nlfmd2338 share the first 318 amino acids, including the two RRM$s$, but differ in their C termini (Fig. 4, A and C). RNAi targeting several different exons respectively showed that only Nlfmd2340 affected female development of BPH, as only the dsNlfmd2340-treated females showed masculinization as demonstrated by smaller bodies, short ovipositors, smaller wings, undeveloped ovaries, and male-specific genital tissues (Fig. 4, B and D, fig. S4, and table S1). Although the external genitalia and testes of dsNlfmd2340-treated males developed

Table 1. Influence of Nlfmd on female and male fertility (offspring number).

| Treatment        | #1   | #2   | #3   | Average |
|------------------|------|------|------|---------|
| dsGFP ♀ X dsGFP ♂ | 238  | 134  | 208  | 193 ± 38 |
| dsNlfmd ♀ X dsGFP ♂ | 0    | 0    | 0    | 0       |
| dsGFP ♀ X dsNlfmd ♂ | 155  | 206  | 200  | 187 ± 20 |

Fig. 1. Nlfmd, an SR-type protein-coding gene, is required for female development. (A) Two splice isoforms of Nlfmd. (B) Motifs and structures of Nlfmd-F. (C) Differential expression of Nlfmd-F and Nlfmd-C in males and females tested using primers designed from constitutive exons. (D) Nlfmd knockdown with the third instar nymphs resulted in masculinization of females, whereas no apparent effect was observed in males. (E) Knockdown of Nlfmd in the third instar nymphs resulted in undeveloped ovaries in females but did not influence the testes development in males. (F) Knockdown of Nlfmd decreased the relative level of the female-specific Nldsx-F in females. (G) Knockdown of Nlfmd resulted in the male-specific Nldsx-M in females. Arrows in (A) indicated the primers designed to test the two main transcripts’ expression. BPHs in (F) and (G) were treated with dsGFP and dsNlfmd in the third instar, and the expression of three biological replicates was done (every replicate includes five to six BPHs). Data are represented as means ± SEM, and Student’s t test was used (**P < 0.001).
normally, the males became infertile with mostly dead sperms in testes, indicating that Nlfmd2 340 is required in males for spermatogenesis and fertility (Fig. 4D). Therefore, Nlfmd2 340 is important in both sexes for aspects related to reproduction. Knockdown of dsNlfmd2 340 in the third instar BPH by RNAi resulted in the expression of the male-specific Nldsx-M in females but did not reduce the female-specific Nldsx-F (Fig. 4, E and F). This is consistent with the observation that Nlfmd knockdown females showed a lesser degree of masculinization than Nlfmd knockdown females.

Nlfmd2 340 transcripts also had a maternal contribution, and they were detected in all developmental stages tested and ubiquitous in both sexes (figs. S5, B and C, and S11). Maternal RNAi knockdown of Nlfmd2 in newly emerged females reduced the number of eggs to less than 10% of that of the dsGFP-treated controls, none of the eggs hatchet, and the eggs showed embryonic arrest (Table 2 and figs. S9B and S12B-b1). CRISPR-Cas9–mediated knockout of Nlfmd2 produced 12.5 to 15% of embryos showing developmental arrest, as indicated by the lack of normal eye spots. Genotyping such embryonic arrested eggs showed that they had mutations in Nlfmd2 (fig. S12). The remaining fraction of eggs developed normal egg spots, and they had no mutation in Nlfmd2. Together, these results suggest that Nlfmd2 340 played critical roles in embryonic development of both sexes and affected Nldsx splicing.

NIFMD-F and NIFMD2 340 interact and regulate Nldsx pre-mRNA alternative splicing in 293T cells

To further decipher the roles of Nlfmd-F and Nlfmd2 340 in the alternative processing of Nldsx, we constructed a plasmid with the mini-gene of Nldsx under the control of the CMV promoter (p3XFlag-dsx) (see Sequence Data in supplementary file S1). The mini-gene contained a portion of the Nldsx that extends from exon 5 to exon 7. At the same time, the cDNA of Nlfmd-F and Nlfmd2 340 were also cloned into plasmid p3XFlag under the control of the CMV promoter. To avoid the influence of factors of the sex determination pathway in insect cells, we chose human embryonic cells 293T for analysis. When p3XFlag-Nldsx was transfected into 293T cells, both the female-specific isoform of mini-Nldsx pre-mRNA (pmNldsx-F) and the male-specific isoform of mini-Nldsx pre-mRNA (pmNldsx-M) were produced. The amount of pmNldsx-M was approximately 1000 times higher than pmNldsx-F, which suggested that the male-specific mRNA is the default splicing isoform (Fig. 5, A and B).

When p3XFlag-Nldsx and p3XFlag-Nlfmd were cotransfected into 293T cells, the level of pmNldsx-F increased by approximately twofold, which suggested that the NIFMD-F protein, which did not have an RNA binding motif, might work together with an unknown protein or protein complex in 293T to enhance the female-specific splicing of the mini-Nldsx pre-mRNA (Fig. 5C). When we transfected
p3XFlag-Nldsx with p3XFlag-Nlfmd2340, the amount of male-specific pmNldsx-M mRNA decreased, and an intermediate splicing isomorph named pmNldsx-M+F-1 was produced, which consisted of an additional 157 bp of exon 6f 3′-terminal sequences (Fig. 5C). However, the amount of female-specific dsx mRNA showed no change (Fig. 5C, c1 and c3). When p3XFlag-Nldsx was cotransfected with both p3XFlag-Nlfmd2340 and p3XFlag-Nlfmd-F, the level of male-specific mRNA pmNldsx-M decreased and the level of female-specific mRNA increased by fourfold (Fig. 5C). In addition to pmNldsx-M+F-1, another new intermediate splicing isomorph named pmNldsx-M+F-2 was also produced, consisting of exon 5, exon 6f, and exon 7m (see Fig. 5C, fig. S14A, and Sequence Data in supplementary file S2). Moreover, when pmNldsx-M+F-1 was cotransfected with both p3XFlag-Nlfmd2340 and p3XFlag-Nlfmd-C, only pmNldsx-M and pmNldsx-M+F-1 were produced, indicating that the non–sex-specific Nlfmd-C was not involved in the regulation of sex-specific splicing of the Nldsx pre-mRNA (fig. S15A). We confirmed that pmNldsx-M+F-1 and pmNldsx-M+F-2 also existed in vivo in BPH at low levels (fig. S14).

To investigate whether NIFMD-F can form a complex with NIFMD2340, immunoprecipitation (IP) and pull-down were performed. These two proteins can be pulled down together (Fig. 5, D and E, and fig. S15B), supporting the hypothesis that NIFMD-F and NIFMD2340 work together to regulate Nldsx alternative splicing.

The (G/A)AAGAA repeats act as the ESEs in regulating Nldsx splicing in 293T cells

Two types of repetitive sequences (C/U)AU(C/A)U(C/U/G)(U/G) A(C/U) (e-value, 2.8 × 10^-6) and (G/A)AAGAA (e-value, 1.6 × 10^-5) were found in a 202-bp region in the female-specific exon 6 by using the MEME Suite (Fig. 6A; http://meme-suite.org/tools/meme) (31). To investigate the cis-required elements in Nldsx pre-mRNA splicing, we first deleted the 202-bp sequence and repeated the transfection experiments in 293T cells (Fig. 5, A and B). Without the 202-bp sequence, the female-specific Nldsx mRNA disappeared, while the amounts of male-specific Nldsx mRNA increased. Moreover, transfection of p3XFlag-Nlfmd-F and p3XFlag-Nlfmd2340 had no influence on sex-specific mRNA splicing without the 202-bp sequence, and no new splice isomorph was produced (Fig. 5, B and C, c1, c2, and c3). These results suggested that there were ESEs in the female-specific exon 6, which were necessary for the regulation by NIFMD/NIFMD2.
We replaced the previously identified repeat sequences with their reverse complementary sequences as controls to study their involvement in the regulation of splicing (Fig. 6B). When the (C/T) AT(C/A)T(C/T/G)(T/G)A(C/T) repeats were replaced by (A/G) T(C/A)(C/A/G)A(T/G)AT(A/G), no influence was found on the regulation of Nldsx pre-mRNA alternative splicing by NlFMD-F and NlFMD2340. However, when (G/A)AAGAA was replaced with TTCTT(T/C), the regulation of NIFMD-F and NIFMD2340 was interfered and pmNldsx-M became the main splicing isoform (Fig. 6C). Moreover, we used microscale thermophoresis (MST) to quantify the affinity between NlFMD2340 and the two repetitive sequences, CAUCUGUAC or GAAGAAGAA, respectively (34). NIFMD2340 could interact with GAAGAAGAA (dissociation constant, $K_D = 1.6366 \pm 0.49644 \mu M$), while no obvious
interaction was found between NIFMD2\(^{340}\) and CAUCUGUAC (see Fig. 6D and supplementary file S2). As expected, protein NIFMD-F was unable to bind GAAGAAGAAA (Fig. 6D). However, the presence of NIFMD-F lowered the affinity (\(K_D = 5.5995 \pm 1.3 \mu M\)) of NIFMD2\(^{340}\) to GAAGAAGAAA, while the presence of GFP did not influence the affinity (\(K_D = 1.709 \pm 0.47464 \mu M\)), consistent with the interpretation that NIFMD interacted with NIFMD2\(^{340}\), resulting in a change in conformation that decreased the binding affinity (Fig. 6D) (35).

**DISCUSSION**

In this study, we presented evidence suggesting that \(Nlfmd\) is a feminizing switch in a hemipteran rice pest BPH. Two splice isoforms of \(Nlfmd\) are present, the female-specific \(Nlfmd\)-F and the non–sex-specific \(Nlfmd\)-C. \(Nlfmd\)-F transcripts are maternally deposited, and the zygotically transcribed \(Nlfmd\)-F are also detected in the early embryo before the presence of the female-specific \(Nldsx\) (Fig. 2A and fig. S8B). RNAi-mediated knockdown of either \(Nlfmd\) or the female-specific \(Nlfmd\)-F in the third instar nymphs resulted in clear somatic masculinization of genetic females (Fig. 1D and figs. S2 and S4). Moreover, \(Nlfmd\) knockdown significantly reduced the level of the female-specific isoform of doublesex (\(Nldsx\)), while it increased the male-specific isoform (Fig. 1, F and G). Together, we have shown that \(Nlfmd\) is a feminizing switch that regulates sex determination by modulating the sex-specific splicing of \(Nldsx\). Complete female-to-male conversion was not observed in the above-mentioned RNAi experiment, which is expected as female differentiation had already progressed to the third instar before RNAi was performed. Thus, we attempted to deplete \(Nlfmd\) at an early developmental stage by performing maternal RNAi and CRISPR–Cas9–mediated knockout (Figs. 2C and 3, B and D). In both cases, depletion of \(Nlfmd\) in early embryos resulted in female-specific embryonic lethality instead of female-to-male conversion. This is consistent with an increasing number of examples showing that sex-specific lethality is observed when the master switch of sex determination is perturbed early in development, as a result of the dual functions of the switch gene in both sex determination and X chromosome dosage compensation (36–39). In contrast, manipulation of sex-switch genes can result in complete sex conversion in organisms for which X chromosome dosage compensation is presumably not needed as in organisms with either homomorphic sex chromosomes or a gene-poor X chromosome (5, 6, 17). In this context, it is relevant to point out that there are more than 1400 genes on the BPH X chromosome, indicating a likely requirement for X chromosome dosage compensation (30, 40). We also note that the production of male-only progeny through the manipulation of \(Nlfmd\) may have practical applications for the control of this devastating agricultural pest.

RNAi-mediated knockdown of the \(Nlfmd\) homologs in \(S. furcifera\) and \(L. striatellus\), which belong to two other hemipteran genera, also masculinized the females (fig. S7), indicating that \(fmd\) may function as the feminizing switch in diverse species within the order Hemiptera. Although a BLASTP search at the default cutoff in NCBI failed to identify any homologs other than what is reported in this manuscript, the putative autoregulatory domain of NIFMD-F showed some similarity to the hymenopteran protein AmFem (brown box in Fig. 1B). The overall identity between NIFMD-F and several TRA family proteins including AmFem ranges from only 5.34% to 8.09%. The order of the three domains—an SR-rich domain, a Pro-rich domain, and a putative 27–amino acid autoregulatory domain—is not conserved between NIFMD and these TRA family proteins (33).\(^\text{35}\)

**Table 2. Maternal dsNlfmd\(^{240}\) affect the embryo development (offspring number).**

| Treatment                           | #1   | #2   | #3   | Average |
|-------------------------------------|------|------|------|---------|
| **dsgfp\(^{♀}\) X dsgfp\(^{♂}\)**  |      |      |      |         |
| Nymphs                              | 238  | 134  | 208  | 193 ± 38|
| Dead eggs                           | 29   | 27   | 21   | 26 ± 3  |
| **dsNlfmd\(^{240}\) X dsgfp\(^{♂}\)**|      |      |      |         |
| Nymphs                              | 0    | 0    | 0    | 0       |
| Embryo arrested eggs                | 14   | 29   | 22   | 17 ± 3  |

This table has the same control with Table 1.

Like TRA, the NIFMD protein is an SR-rich protein that does not have a predicted RNA binding domain. Thus, an RNA binding protein partner is likely required for NIFMD function. It is not yet clear whether NITRA2, the BPH ortholog of the known TRA partner in many other insects, participates in NIFMD function (41). However, we have gathered evidence suggesting that NIFMD2, an hnRNP40/SQID family protein, may serve as one of the RNA binding protein partners of NIFMD. First, \(Nlfmd\) is also involved in sex determination as knockdown of \(Nlfmd\) in the third instar nymphs conferred masculinization, albeit to a lesser extent than the knockdown of \(Nlfmd\) (Fig. 4B and figs. S2 and S4). This masculinization is associated with an increase in the male-specific isoform of \(Nldsx\) without affecting the female-specific \(Nldsx\) (Fig. 4, E and F). This less pronounced masculinization may result from insufficient suppression of \(Nlfmd\) and/or the existence of functionally redundant NIFMD partners (41). Second, MST analysis showed that NIFMD2\(^{340}\) but not NIFMD-F binds the RAAGAA repeat motif in the \(Nldsx\) pre-mRNA, which is required for the production of the female-specific \(Nldsx\) transcript in the 293T cells, with the affinity \(K_D = 1.6366 ± 0.49644 \mu M\) (Fig. 6D). The RAAGAA repeat motif is different from the TCWWCAATCAACA ESE recognized by the
Fig. 5. Effect of Nlfmd-F and Nlfmd2^340 products on the splicing patterns of Nldsx and Nldsx-202 pre-mRNA in 293T cells. (A) Mini-genes and its default splicing in 293T cells. Boxes and the lines between boxes represent the exon and intron sequences, respectively. In the p3XFlag-Nldsx-202, a region of 202 bp was deleted (indicated by black box), and the sequence was listed below in gray box from the female-specific exon 6f. (B) Copy number of pmNldsx-F and pmNldsx-M by absolute quantification in real-time qPCR. (C) The splicing of p3XFlag-Nldsx and p3XFlag-Nldsx-202 was regulated differently by Nlfmd-F and Nlfmd2^340. (c1) The specific primers of pmNldsx-F and pmNldsx-M were used to test the splicing changes by semiquantitative RT-PCR. (c2) The grayscale value of the pmNldsx-M, pmNldsx-M+F-1, and pmNldsx-M+F-2 bands was measured by software Image J. (c3) The expressions of Nldsx-F were measured by absolute quantification with primer pmNldsx-F. (c4) Different model of Nldsx alternative splicing in 293T cells. Naming of samples 1 to 8 is consistent in (c1), (c2), and (c3). (D) NIFMD-Flag immunoprecipitated (IP) with Nlfmd^2340. (E) NIFMD-Flag pull-down assay with Nlfmd^2340-Mbp. Data in (B) are represented as means ± SEM. Student's t test was used in (B) (**P < 0.001).
D. melanogaster TRA2 but similar to an A-rich repeat motif predicted to be the ESE in SQUID-mediated sex-specific splicing in D. melanogaster (13, 42). We note that some of the SQUID-mediated sex-specific splicing is independent of the SXL-dependent pathway in D. melanogaster (42). Third, in vitro experiments suggest that NIFMD2340 and NIFMD-F may interact. In the vertebrate 293T cells, NIFMD2340 and NIFMD-F formed a protein complex according to antibody pull-down assays, and they appear to work synergistically to increase the female-specific Nldsx splicing and alter the male-specific Nldsx splicing (Fig. 5C). There is also an indication from the MST analysis that NIFMD-F may alter the binding affinity of NIFMD2340 to the RAAGAA repeat motif (35). In summary, we have presented evidence suggesting that NIFMD2 is involved in sex determination in BPH, and the SQUID-like protein NIFMD2340 binds a putative ESE in the Nldsx pre-mRNA and may interact with NIFMD-F to modulate Nldsx splicing in vitro. Future experiments are needed to determine whether NIFMD2 functions in vivo as an RNA binding partner of the feminizing switch NIFMD.

On the basis of the above results, we propose a model for the sex determination pathway of N. lugens (Fig. 7). The female-specific Nfmd-F functions as the feminizing switch, and it is either activated by a double dosage of the X chromosome in females or inhibited by a male-determining factor on the Y chromosome in males. Both models have been demonstrated in dipteran insects: while the XSE proteins act as the primary signal in D. melanogaster, a Y chromosome gene CWC22 in the house fly acts as the male determiner by inhibiting the Mdtra (3, 4). We have no direct evidence in BPH to support or rule out either of the two models. However, we note that fmd also functions as the feminizing switch in S. furcifera and L. striatellus, two Delphacidae relatives of N. lugens that use the XX
be determined whether NlTRA2 is also involved in the NlFMD-RNA binding partners of NlFMD-F. We also note that it remains to mediated sex-determination pathway.

G/AAGAA in the female-specific exon 6. With NlFMD-F to promote the splicing of female-specific

Fig. 7. A proposed model for sex determination in BPH. (A) Model for sex determination pathway of BPH. In female of BPHs, dsx alternative splicing process is controlled by three upstream genes, Nlfmd2, d and Nlfmd2. Nlfmd2 needs the help of Nlfmd2 to maintain the female-specific dxf-s x splicing and inhibit Nldsx-M production. A primary signal is suspected to be activated by double X chromosomes in females to regulate gene Nlfmd to produce Nlfmd-F, or a Y chromosome factor interferes functional female-specific Nlfmd-F in males. (B) Model for the molecular mechanism of Nldsx pre-mRNA sex-specific alternative splicing. Protein Nlfmd2 cooperates with Nlfmd-F to promote the splicing of female-specific Nldsx-F, and they bind on (G/A)AAGAA in the female-specific exon 6.

(female) and XO (male) system for sex determination (30). Therefore, we speculate that it is possible that conserved XSE(s) may serve as the primary signal in all three Delphacidae species. We also propose that the non–sex-specific Nlfmd2 binds the RAAGAA repeat in the Dlx pre-mRNA and assists the feminizing switch Nlfmd-F in promoting the female-specific splicing of Nldsx. In vivo support is still needed to confirm that Nlfmd2 is one of the RNA binding partners of Nlfmd-F. We also note that it remains to be determined whether NlTRA2 is also involved in the Nlfmd-mediated sex-determination pathway.

MATERIALS AND METHODS

Insect rearing

The BPH populations used here were collected in Hangzhou (30°16′9 N, 12°11′ E), China, in 2008. BPHs were reared at 26° ± 0.5°C on rice seedlings (Xiushui 128) under a 16-hour light:8-hour dark photoperiod.

Transcriptome sequencing and RNAi screening

More than 100 mated BPH females were allowed to lay eggs on rice seedlings for 1 hour. BPHs were then removed, and the eggs (about 200) in the rice seedling were collected at the time point of 0, 24, and 72 hours. Three replicates were carried out for each time point. Total RNA of the collected eggs was extracted using TRIzol reagent (Takara, Dalian, China) following the manufacturer’s instructions. Transcriptome sequencing was performed on the Illumina HiSeq platform (Illumina, San Diego, USA). Raw reads were quality trimmed and assembled de novo with Trinity software. Bioinformatics analysis of annotation was performed as described previously (43). We focused on genes that are highly expressed in the earlier embryos that are either with an unknown function or contain RRMs. Approximately 200 genes met these criteria and were chosen for RNAi screening.

The sequence analysis and alignments

The Splign tool from NCBI (www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi) was used to predict the exons and introns of Nlfmd by aligning the sequences of the RT-PCR products with the genomic sequence of BPH (PRJNA177647). The alignment of fmd homologs was generated using Clustal X (44) and GENEDOC (45).

RNAi interference

The common region or specific region of Nlfmd and Nlfmd2 isoforms was used as DNA templates for dsRNA synthesis. The MEGAscript T7 High Yield Transcription Kit (Ambion, catalog no. AM1334) was used to synthesize the dsRNAs according to the manufacturer’s instructions. The concentration of the product was quantified using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA). Afterward, we followed the BPH dsRNA treatment method described by Xue et al. (46).

PCR of male-specific genomic DNA fragment

Genomic DNA was purified from individual dsRNA-treated adult planthoppers or offspring adults of maternal RNAi females using the Wizard Genomic DNA Purification Kit according to the manufacturer’s instructions (Promega, Madison, WI). The DNA concentration was measured and diluted to 50 ng/ml. Primers for a male-specific sequence were reported by Kobayashi and Noda (47). PCR was performed on 1 µl of genomic DNA in a 25-µl reaction mixture (50 U/µl Taq DNA polymerase with Mg2+ buffer for DNA polymerase, Biocolors, Shanghai, China; 2 mmol dNTPs, TaKaRa). Thirty cycles of amplification were performed, each of which consisted of denaturation for 30 s at 94°C, annealing for 15 s at 56°C, and extension for 30 s at 70°C. Samples of the PCRs were analyzed on 2% agarose gels.

qRT-PCR analysis

Total RNA was first isolated from the BPH whole bodies or tissues using RNAiso Plus (TaKaRa). RNA sample (1 µg of total RNA) was reverse transcribed using the PrimeScript first-strand cDNA synthesis kit (TaKaRa, catalog no. 4,110A). The internal control for quantitative RT-PCR (qRT-PCR) was the 18S rRNA gene of BPH, and the SYBR Premix Ex Taq Kit (TaKaRa) was used for qRT-PCR. The relative quantitative method was used to evaluate quantitative variations (48).

Semiquantitative RT-PCR

Total RNA was extracted from embryos, first-, second-, third-, fourth-, fifth-instar nymphs, and female and male adults, respectively; 1 µg of RNA was used to perform reverse transcription in 20-µl reactions using Quant reverse transcriptase (TsINGEN, Beijing, China) according to the manufacturer’s instructions, diluted 10 times; and 1 µl was used in subsequent PCRs. The total RNA of individual eggs at different embryonic stages was extracted using RNAiso Plus (TaKaRa), and then 50 ng of RNA was used to perform reverse transcription in 5 µl using the Single Cell Sequence Specific Amplification Kit (Vazyme, NanJing, China); the assay pool used in this kit consisted of Nlfmd-F/C, qPCR-N1185, and Nldsx-F primers, and the concentration of each primer was 0.1 μM.
Absolute quantification in qRT-PCR

PCR products of different primers were purified and quantified, the products were serially diluted eightfold, and the logarithm of their initial template copy numbers and the Ct values of the primers in every dilution were used to construct standard curves. The concentrations of the PCR products were measured using a NanoDrop 2000 (Thermo Fisher Scientific, America), and the copy numbers of the PCR products were calculated using the following equation:

\[
\text{DNA (copy)} = \frac{6.02 \times 10^{23} \times \text{DNA amount (g)}}{\text{DNA length (bp)} \times 660 \times \text{mol/dp}} \times (49, 50) \times (1)
\]

The Ct values of the dilutions were obtained by qRT-PCR with different primers. The PCR amplification efficiency (E) of each primer was obtained using the Bio-Rad CFX manager 3.1 (Bio-Rad Laboratories). Ideally, the value of E ranges from 90 to 110% (fig. S13).

Dissection and fertility analysis

To study the systemic expression of NlFmd-F and NlFmd2340 in BPHs, the tissues such as head, muscle, wing, fat body, gut, integument, ovary, or testes were dissected from 2-day-old fifth instar females (n = 100) and males (n = 100), and the sex of nymphs were identified by gonotome difference (51). Total RNA of the collected tissues was extracted using TRIzol reagent (Life Technologies, USA) according to the manufacturer’s instructions.

Third instar nymphs were injected with dsGFP or dsNlFmd, and then were dissected or used for fertility analysis 3 days after emergence. The treated BPH pairs were reared on rice seedlings, and the number of offspring and eggs was examined 10 or 5 days after deposition. Each pair of adults were reared on the same kind of rice seedling.

Sperm viability

The LIVE/DEAD Sperm Viability Kit (L-7011) (Invitorgen, America) was used to measure sperm activity according to the manufacturer’s protocol. The semen was released by cutting open the vas deferens with dissection scissors in 300 µl of buffer [10 mM Hapes, 150 mM NaCl, and 10% bovine serum albumin (pH 7.4)]. Then, 5 µl of diluted SYBR14 dye was added and incubated for 10 min at 36°C. Next, 5 µl of propidium iodide was added to 300 µl of buffer and incubated for another 10 min. Last, the number of green sperm (live) and red sperm (dead) in a 20-µl sample was counted using a Zeiss LSM 780 confocal microscope (Carl Zeiss Microlmaging, Göttingen, Germany). Each semen sample represented 10 individual insects, and each treatment was pooled from three independent experiments.

Image processing

DsrNA used in this study was injected into the third instar nymphs; the phenotypes after emergence were recorded using a DFC320 digital camera attached to a LEICA S8APO stereomicroscope.

Maternal RNAi and offspring counting

Virgin female adults collected 12 hours after emergence were injected with dsGFP and dsNlFmd and dsNlFmd2340, reared for 3 days, and then mated with wild-type males. Each pair were reared separately. The offspring were reared, and numbers of each sex were counted. The dead eggs were dissected and counted from the seedlings after no larva hatching for three consecutive days.

CRISPR-Cas9

Sequences of NlFmd-F (GenBank accession number MW082042) and NlFmd2340 (GenBank accession number MW082037) and BPH genomes (GenBank accession number PRJNA177647) were used to search single guide RNAs (sgRNAs) used in CRISPR-Cas9 by searching single guide RNAs (sgRNAs) used in CRISPR-Cas9 by the sgRNAcas9 algorithm (52), and two sgRNAs with lowest off-target possibility were predicted to target NlFmd-F and NlFmd2340, respectively. The sgRNA was prepared using T7 High Yield RNA Transcription Kit (Vazyme, China), the sense primer of sgRNA contained a T7 polymerase-binding site, and the antisense primer contained a partial sgRNA sequence that was complementary to pMD19-T sgRNA scaffold vector. Cas9 mRNA was in vitro transcribed from plasmid pSP6-2sNLS-Scas9 vector and purified using the mMESSAGE mMACHINE SP6 Transcription Kit (Thermo Fisher Scientific) and Poly(A) Tailing Kit (Thermo Fisher Scientific). The microinjection was performed according to the report by Xue et al. (28), and the eggs within 1 hour of oviposition were used. At 48 hours after injection, genomic DNA was extracted from injected eggs with the Wizard Genomic DNA Purification Kit (Promega), and the mutation of the sgRNA target sites was tested by primers and sequenced. The DNA of male wings in G0 were extracted and tested, and then the mutated males were mated with wild-type females to produce G1. In G1, the mutatd males were mated with mutatd females to produce G2, and the number of males and females in G2 was counted; some of them were used for genotyping the mutation, and the eggs of G1 that failed to hatch were counted and recorded.

Cell culture and transfection

Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco’s Modified Eagles Medium (DMEM) that was supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a humidified incubator that contained 5% CO2. p3XFlag-CMV and pcDNA3.1(+)myc-His C constructs were transfected into HEK293T cells by PolyJet, SignaGen, USA, according to the manufacturer’s instructions. Thirty-six hours after transfection, cells were collected and the next experiment was performed.

Molecular cloning and plasmid construction

NlFmd-F, NlFmd-C, NlFmd2340, Mini-Nldsx, and the mutants were amplified by PCR and cloned into p3XFlag-CMV for the mini-Nldsx system; NlFmd2340 was also cloned into pcDNA3.1(+)myc-His C and pMAL-p5x for the IP and pull-down assays, respectively.

Microscale thermophoresis

RNA of sequences CAUCUGUAC and GAAGAA were labeled with fluorescent dye cyanine 3 with the help of company GenScript, China. Subsequently, interaction of labeled RNAs to purified protein NIFMD2340 (50 µM, prokaryotic expression) was measured in PBS. The concentration of labeled RNA was constant at 20 nM; purified protein NIFMD2340 was added in a serial dilution (PBS) at a final concentration between 25 and 0.000763 µM. Samples were loaded into Monolith NT.115 system using 20% excitation power and 40% MST power to determine Kd values. Data were analyzed using MO.Affinity Analysis software version 2.2.4.

Purified protein NIFMD-F (2.5 µM, Eukaryotic expression) and GFP (2.5 µM, prokaryotic expression) were added to NIFMD2340 (50 µM) (1:1), the complex was added in a serial dilution (PBS) at a final concentration between 25 and 0.000763 µM, and the concentration of labeled GAAGAA was constant at 20 nM. Samples
were loaded into the Monolith NT.115 system using 20% excitation power and 40% MST power to determine Kp values. Data were analyzed using MO.Affinity Analysis software version 2.2.4. The binding between NIFMD-F and labeled GAAGAA was also tested.

**IP and pull-down assays**

For IP, cells were collected after washing three times in cold PBS [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, and 1.4 mM KH2PO4 (pH 7.4)] and lysed with buffer [25 mM tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40; protease and phosphatase inhibitors added before use]. The lysate was centrifuged and immunoprecipitated with Anti-DYKDDDDK IP Resin (Genscript, China). Precipitated proteins as well as initial whole-cell lysates were boiled with SDS loading buffer at 100°C for 10 min.

For pull-down assays, purified Flag-fused proteins or Mbp-fused expressed in HEK293T cells or Escherichia coli was mixed with Anti-DYKDDDDK G1 Affinity Resin (Genscript, China) or Amylose Resin beads (NEB, USA) for 16 hours at 4°C, respectively. After extensive washing with PBS, the beads were incubated with purified Mbp-tagged fusion protein at 4°C for 16 hours and washed with tris-buffered saline [50 mM tris-HCl and 150 mM NaCl (pH 7.4)]. The beads as well as initial whole-cell lysates were then boiled with SDS loading buffer at 100°C for 10 min.

All the precipitated proteins were separated by SDS–polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, incubated with primary (1:10,000) and secondary (1:5000) antibodies, and detected with an enhanced chemiluminescence staining kit (fig. S7). The antibody used in these assays were the following: anti-Flag M2 monoclonal antibody (Sigma-Aldrich, F3165, USA), THE His Tag Antibody, mAb, Mouse (Genscript, China), and anti-MBP monoclonal antibody (NEB, USA).

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at https://science.sciencemag.org/lookup/doi/10.1126/sciadv.abf9237

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Competing interests: The authors declare that they have no competing interests.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Sequences of Nlfmd-C, Nlfmd-D, Nlfmd2385, Nlfmd2386, and Nlfmd242 were deposited in GenBank with accession numbers MW082042, MW082041, MW082037, MW082037, and MW082039/MW082040, respectively.

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