BIOCHEMISTRY

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 determiner* (*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 determiner 2* (*Nlfmd2*) also conferred masculinization. In vitro experiments showed that an *Nlfmd2* isoform, NlFMD2^{340}, bound the RAAGAA repeat motif in the *Nldsx* pre-mRNA and formed a protein complex with NlFMD-F to modulate *Nldsx* splicing, suggesting that NlFMD2 may function as an RNA binding partner of the feminizing switch NlFMD. 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 *CWC22* (*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 *Anopheles gambiae*, *gYG2/Yob* is the maleness gene (*7*, *8*). In the honey bee *Apis mellifera*, *csd* acts as the primary signal according to its allelic composition (*9*). In *Bombyx mori*, a single female-specific piwi-interacting RNA is the primary sex determinant (*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* (*Dmsxl*) 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) $(TC(T/A)(T/A)CAATCAACA)_6$ 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 *sxl* homolog *Nlsxl* 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 prolixus* (*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* (*Nlfmd*) and *Female determinant factor 2* (*Nlfmd2*), respectively.

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We present evidence suggesting that *Nlfmd-F*, the female-specific isoform of *Nlfmd*, is a feminizing switch that modulates sex-specific *Nldsx* splicing. Our work yielded new insight into the RNA binding protein partner and the ESE associated with the feminizing switch. *NlFmd* homologs that showed similar functions were identified from two other hemipteran species, *S. furcifera* and *L. striatellus*, suggesting a conserved role in several hemipteran species.

RESULTS

Nlfmd encodes a Ser/Arg-rich (SR-rich) protein required for female development and sex-specific alternative splicing of *Nldsx*

To discover candidate genes that are involved in sex determination, we performed a large-scale screen targeting more than 200 *N. lugens* embryonic transcripts (supplementary file S1) by injecting their corresponding double-stranded RNAs (dsRNAs) in the third instar nymphs of *N. lugens*. A gene *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), *Nlfmd* has two isoforms, a female-specific *Nlfmd-F* (GenBank accession number MW082042) and a non–sex-specific *Nlfmd-C* (GenBank accession number MW082041) (Fig. 1A). *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. *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 *Nlfmd* showed some similarity to the hymenopteran protein *A. mefillera feminizer* (AmFem) (brown box in Fig. 1B). However, the overall protein sequence identity between 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 NlFMD and these TRA/FEM family proteins (*33*).

Having characterized the isoforms of *Nlfmd*, we performed further RNAi experiments using a dsRNA that targets the common region (exons 1, 2, 3, and 4) of the two *Nlfmd* transcripts (fig. S1A). The results of the RNAi showed that all females with RNAi knockdown of *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 *Nlfmd* was systemic (Fig. 1, D and E, figs. S2 and S3, and table S1). However, knockdown of *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 *Nlfmd-F*–specific exon 14 into the third instar nymphs resulted in the same phenotypes compared to the knockdown of the common regions of *Nlfmd-F* and *Nlfmd-C* (fig. S4).

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

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

Nlfmd-F is transcribed before the appearance of *Nldsx-F* in early embryos, and *Nlfmd* depletion results in female-specific embryonic lethality

Transcripts of *Nlfmd* could be detected by RT-PCR in all developmental stages starting from newly oviposited eggs, indicating a maternal contribution. However, the expression of *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 *Nlfmd* transcripts in individual eggs at different embryonic development stages, and the results showed that zygotic *Nlfmd-F* was established between 12 and 24 hours before the initial detection of the female-specific *Nldsx-F* between 48 and 60 hours after oviposition (fig. S8). In addition, *Nlfmd-F* transcripts can be detected in all tissues tested in adult female BPHs (fig. S5A).

Knockdown of *Nlfmd* by maternal RNAi in newly emerged females reduced the number of eggs to only 10% of that of the ds*gfp*-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 *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 *Nlfmd*. Only heterozygous male mutants were obtained in G_0, according to genotype analysis performed using adult wings (Fig. 3, A and B). No homozygous female mutants were found in the G_2 progeny from a cross between heterozygous G_1 mutant males and females (Fig. 3B-b3). However, a number of eggs that showed embryonic arrest were found in G_2, 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 *Nlfmd* is required for female embryonic development (Fig. 2C). Homozygous G_2 mutant males were found, but the number was less than expected, suggesting that *Nlfmd*, presumably the non–sex-specific *Nlfmd*-C, may be involved in male development (Fig. 3, C and D).

Nlfmd2 also affects female development and *Nldsx* splicing

During our RNAi screen, we also found a second gene that is involved in sex determination in BPH, and we named it *Female determiner 2* (*Nlfmd2*). *Nlfmd2* encodes multiple protein isoforms with two RRMs. They belong to a conserved and widely distributed RNA binding protein family that includes the *D. melanogaster* SQUID protein and other heterogeneous nuclear ribonucleoprotein 40 proteins (hnRNP40), but not the TRA2 family of proteins (fig. S10). Gene *Nlfmd2* had four alternative splicing isoforms in both sexes and potentially encode three different proteins (*Nlfmd2*^{340}, *Nlfmd2*^{338}, and

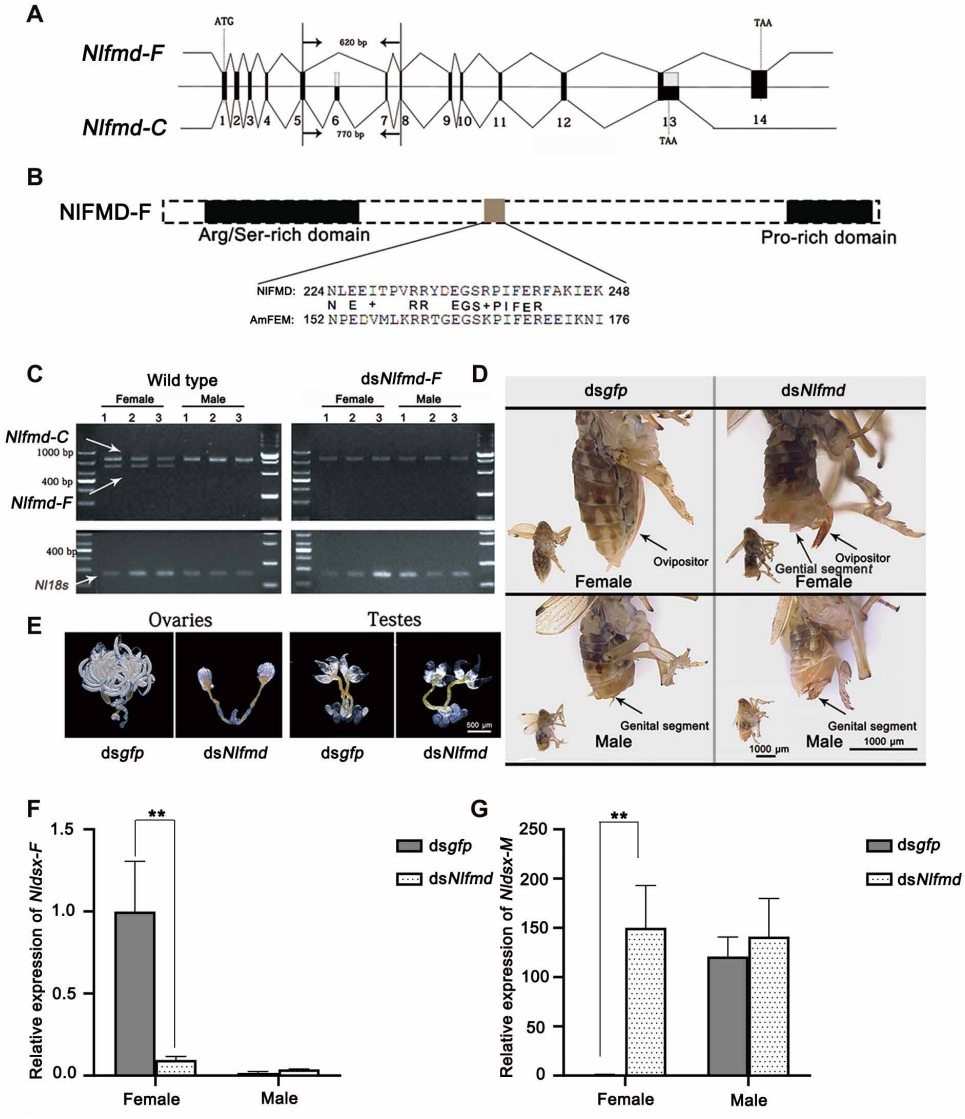


Fig. 1. *Nlfmd*, an SR-type protein-coding gene, is required for female development. (**A**) Two splice isoforms of *Nlfmd*. (**B**) Motifs and structures of *Nlfmd-F*. (**C**) Differential expression of *Nlfmd-F* and *Nlfmd-C* in males and females tested using primers designed from constitutive exons. (**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 ds*gfp* and ds*Nlfmd* 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 (**$P < 0.001$).

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

Treatment	#1	#2	#3	Average
ds*gfp*♀ X ds*gfp* ♂	238	134	208	193 ± 38
ds*Nlfmd*♀X ds*gfp*♂	0	0	0	0
ds*gfp*♀ X ds*Nlfmd* ♂	155	206	200	187 ± 20

Nlfmd2^{62}; GenBank accession numbers MW082037, MW082038, and MW082039/MW082040) (Fig. 4, A and C). In the four transcripts, exon2 and exon3 were retained in *Nlfmd2*^{62}, but spliced out in *Nlfmd2*^{340} and *Nlfmd2*^{338}, and exon 2 contained a stop codon that prematurely terminated the translation of *Nlfmd2*^{62} (Fig. 4A). *Nlfmd2*^{340} and *Nlfmd2*^{338} share the first 318 amino acids, including the two RRMs, but differ in their C termini (Fig. 4, A and C). RNAi targeting several different exons respectively showed that only *Nlfmd2*^{340} affected female development of BPH, as only the ds*Nlfmd2*^{340}-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 ds*Nlfmd2*^{340}-treated males developed

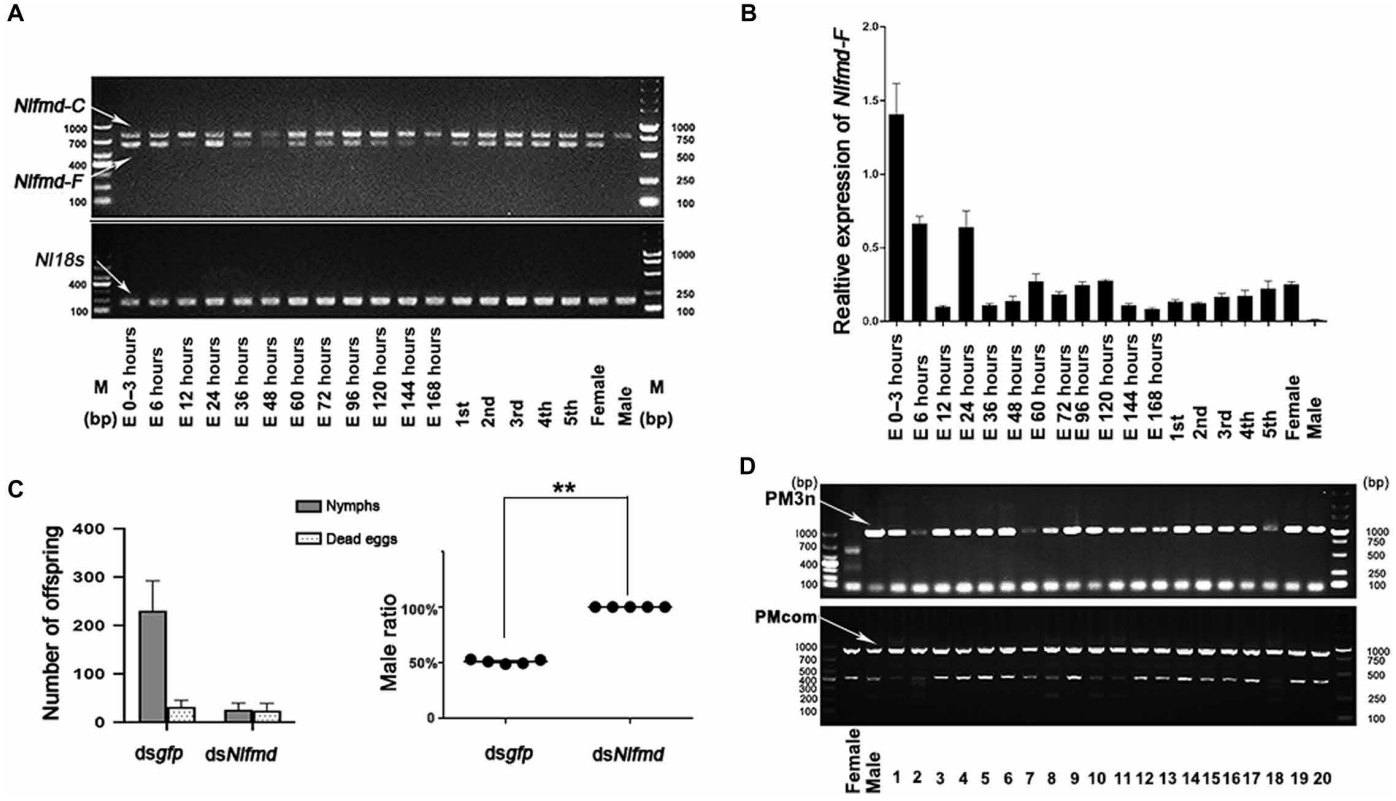


Fig. 2. Transcription profile of *Nlfmd* and the impact of maternal RNAi knockdown of *Nlfmd*. (**A**) Detection of transcripts of *Nlfmd-F* at different developmental stages (E, embryonic; first to fifth: different instars) and in different sexes. (**B**) Relative transcript level of *Nlfmd-F* at different development stages. (**C**) Maternal ds*Nlfmd* affected the females' egg laying, hatching, and the sex ratio of the offspring. (**D**) Offspring genotypes of the females injected with maternal ds*Nlfmd*. After the offspring developed into adults, primer set PM3n and PMcom were used to test their genotypes. Primer set PM3n was used to amplify male-specific sequences, and PMcom was used for common sequences of both sexes. The primers were reported by Kobayashi and Noda (*47*), and we changed the primer name of both sexes from PMFemale to PMcom. PCR results of PM3n primers indicated that all the offspring of ds*Nlfmd*-treated females are XY males, and they had the same genotype as ds*gfp* males (XY). With the help of male-specific primer PM3n and non–sex-specific primer PMcom, all the offspring were confirmed to be males (XY). Data in (B) and (C) are represented as means ± SEM. Student's *t* test was used in (C) (**$P < 0.001$).

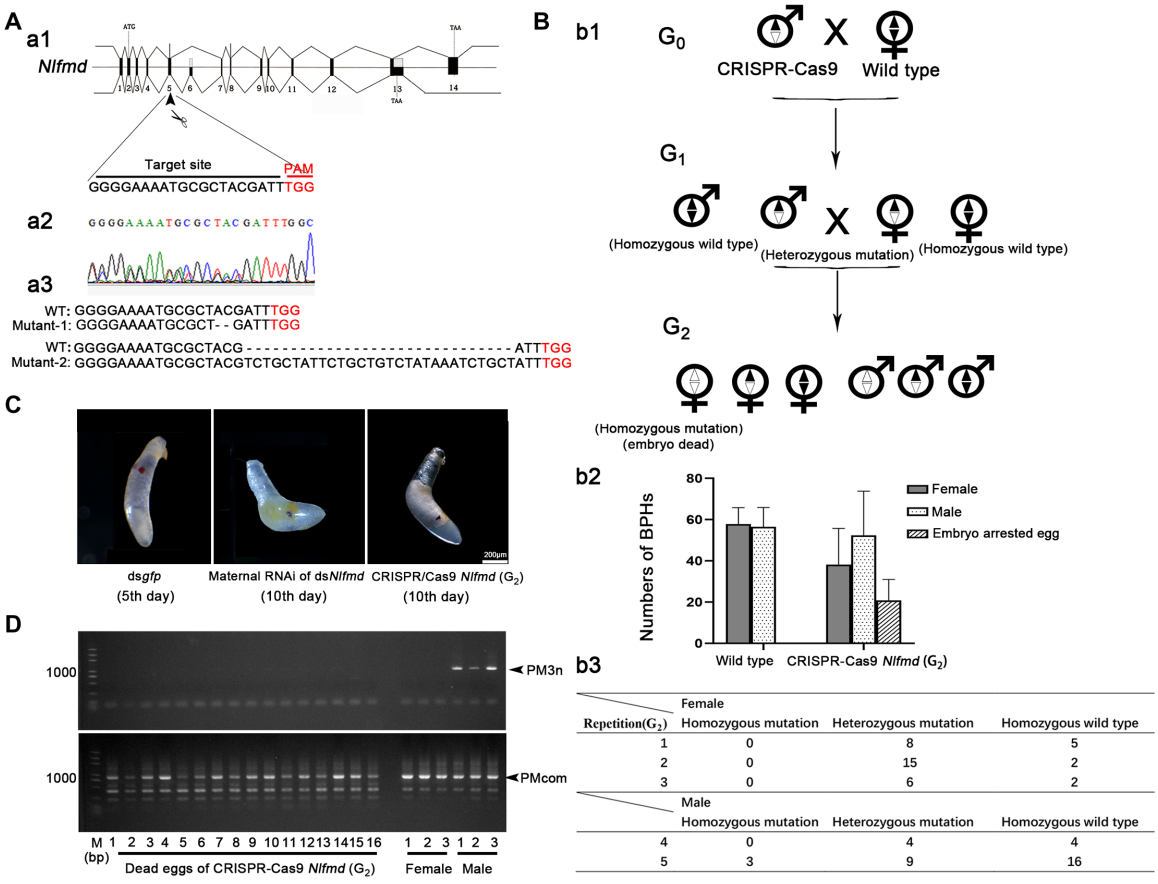
normally, the males became infertile with mostly dead sperms in testes, indicating that $Nlfmd2^{340}$ is required in males for spermatogenesis and fertility (Fig. 4D). Therefore, $Nlfmd2^{340}$ is important in both sexes for aspects related to reproduction. Knockdown of ds$Nlfmd2^{340}$ in the third 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 *Nlfmd2* 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 ds*gfp*-treated controls, none of the eggs hatched, 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.

NlFMD-F and NlFMD2^{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 minigene of *Nldsx* under the control of the CMV promoter (p3XFlag-dsx) (see Sequence Data in supplementary file S1). The minigene 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 (pm*Nldsx-F*) and the male-specific isoform of mini-*Nldsx* pre-mRNA (pm*Nldsx-M*) were produced. The amount of pm*Nldsx-M* was approximately 1000 times higher than pm*Nldsx-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 pm*Nldsx-F* increased by approximately twofold, which suggested that the NlFMD-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



Repetition(G_2)	Female: Homozygous mutation	Heterozygous mutation	Homozygous wild type
1	0	8	5
2	0	15	2
3	0	6	2
	Male		
	Homozygous mutation	Heterozygous mutation	Homozygous wild type
4	0	4	4
5	3	9	16

Fig. 3. CRISPR-Cas9 of *Nlfmd*. (**A**) CRISPR-Cas9–induced mutations at the *Nlfmd* loci. (a1) Schematic diagram of the single guide RNA (sgRNA)–targeted sites in exon 5 of *Nlfmd*. (a2) Sequencing chromatograms of PCR products spanning the target sites in *Nlfmd* G_0 mutants. (a3) Sequences of insertion and deletion flanking the target sites in G_1 mutants. (**B**) Absence of homozygous *Nlfmd* mutant in G_2 female BPHs. (b1) Schematic diagram of CRISPR-Cas9. Three G_0 mutant males were mated with three virgin BPH females, respectively, to produce G_1 BPHs; 10 G_1 mutant males mated with 10 G_1 mutant virgin BPH females, respectively, to produce G_2 BPHs; and 6 of these single-pair families were counted. (b2) Number of females, males, and embryonic arrest eggs in the wild-type (WT) control and G_2. (b3) Random detection of G_2 genotype. No homozygous mutant was found in female BPHs of G_2. (**C**) Embryonic arrested egg of maternal RNAi (ds*Nlfmd*) and G_2. (**D**) Genotype of the G_2 embryonic arrested eggs. The embryonic arrested eggs of one G_2 replicate were tested, and all the eggs were XX and homozygous mutations (the results of genotype sequencing were supplied in Raw data).

p3XFlag-*Nldsx* with p3XFlag-*Nlfmd2*^{340}, the amount of male-specific pm*Nldsx-M* mRNA decreased, and an intermediate splicing isoform named pm*Nldsx-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-*Nlfmd2*^{340} and p3XFlag-*Nlfmd-F*, the level of male-specific mRNA pm*Nldsx-M* decreased and the level of female-specific mRNA increased by fourfold (Fig. 5C). In addition to pm*Nldsx-M+F-1*, another new intermediate splicing isoform named pm*Nldsx-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 p3XFlag-*Nldsx* was cotransfected with both p3XFlag-*Nlfmd2*^{340} and p3XFlag-*Nlfmd-C*, only pm*Nldsx-M* and pm*Nldsx-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 pm*Nldsx-M+F-1* and pm*Nldsx-M+F-2* also existed in vivo in BPH at low levels (fig. S14).

To investigate whether NlFMD-F can form a complex with NlFMD2^{340}, 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 NlFMD-F and NlFMD2^{340} 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-*Nlfmd2*^{340} had no influence on sex-specific mRNA splicing without the 202-bp sequence, and no new splice isoform 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 NlFMD/NlFMD2.

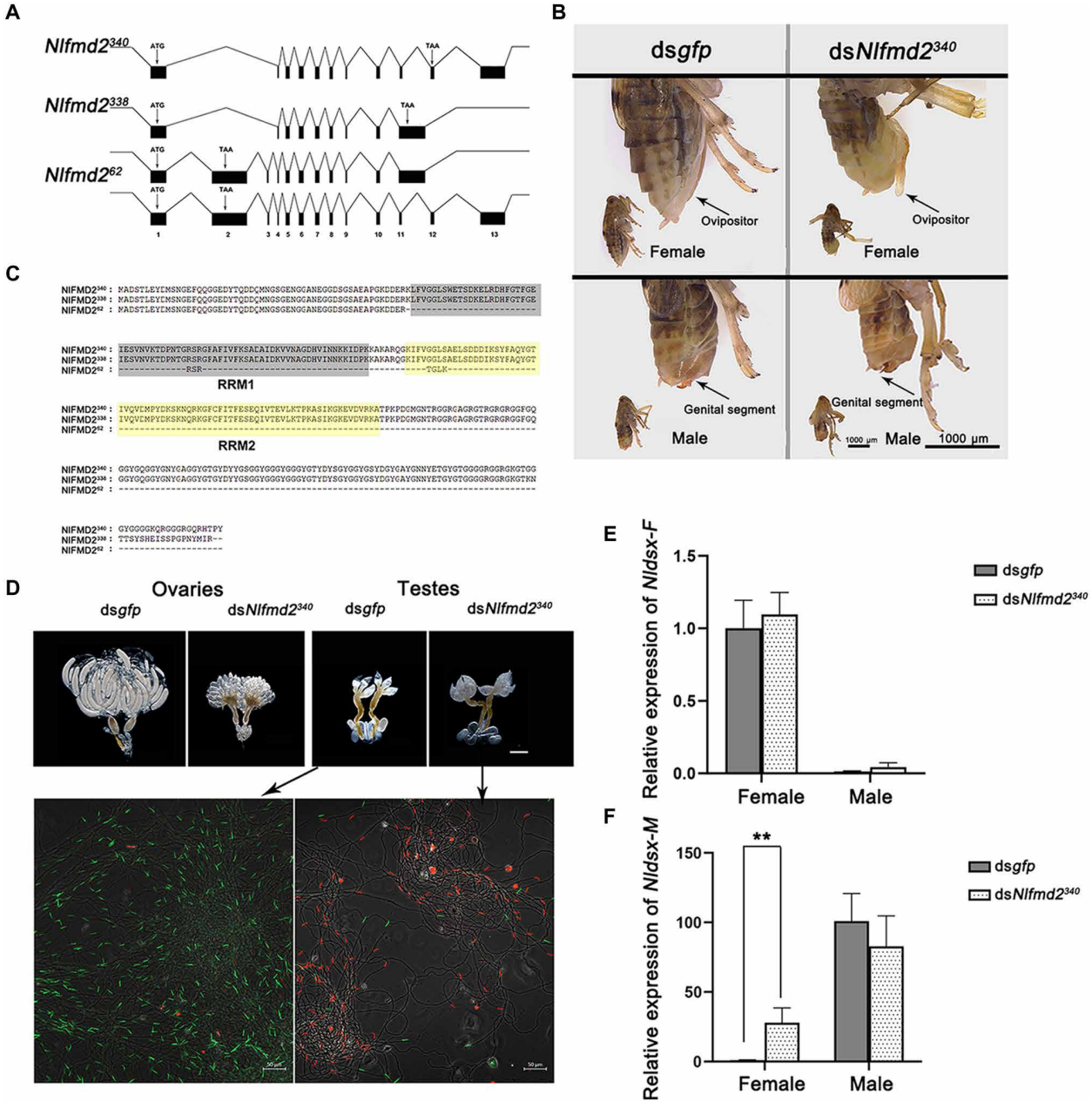


Fig. 4. The influence of *Nlfmd2* knockdown on BPH. (**A**) Splicing diagram of the *Nlfmd2* gene. Start and stop codons are indicated. (**B**) Phenotypes of ds*Nlfmd2*^{340}-treated female and male adults. BPHs of the third instar were treated with ds*gfp* and ds*Nlfmd2*^{340}, respectively. Additional images of the ds*Nlfmd2*^{340}-treated female exhibiting an intersex phenotype are provided in figs. S2A and S4. (**C**) Alignment of the three Nlfmd2 amino acid sequences. Two common RNA recognition motifs (RRMs) of *Nlfmd2*^{340} and *Nlfmd2*^{338}, RRM1 and RRM2, are indicated in gray and yellow, respectively. No RRMs are found in *Nlfmd2*^{62}. (**D**) Influence of *Nlfmd2*^{340} on the development of ovaries and testes. Sperms from ds*gfp*- and ds*Nlfmd2*^{340} male adults 3 days after emergency were tested. Green indicates live sperm stained by SYBR 14 dye, and red indicates dead sperm stained by propidium iodide. (**E**) Knockdown of *Nlfmd2*^{340} had no obvious effect on the level of the female-specific isoform *Nldsx-F*. (**F**) Knockdown of *Nlfmd2*^{340} increased the level of the male-specific isoform *Nldsx-M* in females. Data in (E) and (F) are represented as means ± SEM, and Student's *t* test was used (**$P < 0.001$).

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 NlFMD2^{340}. However, when (G/A)AAGAA was replaced with TTCTT(T/C), the regulation of NlFMD-F and NlFMD2^{340} was interfered and pm*Nldsx-M* became the main splicing isoform (Fig. 6C). Moreover, we used microscale thermophoresis (MST) to quantify the affinity between NlFMD2^{340} and a Cy3 (Cyanine 3)-labeled monomer of the two repetitive sequences, CAUCUGUAC or GAAGAAGAA, respectively (*34*). NlFMD2^{340} could interact with GAAGAAGAA (dissociation constant, K_D = 1.6366 ± 0.49644 μM), while no obvious

Table 2. Maternal ds*Nlfmd2*^{340} affect the embryo development (offspring number).

Treatment		#1	#2	#3	Average
ds***gfp***♀ X ds***gfp***♂	Nymphs	238	134	208	193 ± 38
	Dead eggs	29	27	21	26 ± 3
ds*Nlfmd2*^{340} ♀X ds*gfp*♂	Nymphs	0	0	0	0
	Embryo arrested eggs	14	29	22	17 ± 3

This table has the same control with Table 1.

interaction was found between NlFMD2^{340} and CAUCUGUAC (see Fig. 6D and supplementary file S2). As expected, protein NlFMD-F was unable to bind GAAGAAGAA (Fig. 6D). However, the presence of NlFMD-F lowered the affinity (K_D = 5.5995 ± 1.3 μM) of NlFMD2^{340} to GAAGAAGAA, while the presence of GFP did not influence the affinity (K_D = 1.709 ± 0.47464 μM), consistent with the interpretation that NlFMD interacted with NlFMD2^{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 NlFMD-F showed some similarity to the hymenopteran protein AmFem (brown box in Fig. 1B). The overall identity between NlFMD-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 NlFMD and these TRA family proteins (*33*). *Tra* homologs *Rptra*, *Bgtra*, and *Phtra* were recently reported in three hemimetabolous species: *R. prolixus*, *Blattella germanica* and *Pediculus humanus*. The protein sequence identity between NlFMD-F and RpTRA, BgTRA, and PhTRA is 15.89, 15.41, and 15.28%, respectively. However, NlFMD-F does not have the partial RRM, which was reported in RpTRA, and BgTRA (*32*). Further analysis is needed to determine whether NlFMD is a TRA ortholog, or NlFMD and TRA represent a case of convergent evolution. Regardless the evolutionary history, the characterization of *Nlfmd* significantly extends our understanding of the feminizing switch in hemimetabolous insects.

Like TRA, the NlFMD 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 NlFMD function. It is not yet clear whether NlTRA2, the BPH ortholog of the known TRA partner in many other insects, participates in NlFMD function (*41*). However, we have gathered evidence suggesting that NlFMD2, an hnRNP40/SQUID family protein, may serve as one of the RNA binding protein partners of NlFMD. First, *Nlfmd2* is also involved in sex determination as knockdown of *Nlfmd2* 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 *Nlfmd2* and/or the existence of functionally redundant NlFMD partners (*41*). Second, MST analysis showed that NlFMD2^{340} but not NlFMD-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 μM (Fig. 6D). The RAAGAA repeat motif is different from the TCWWCAATCAACA ESE recognized by the

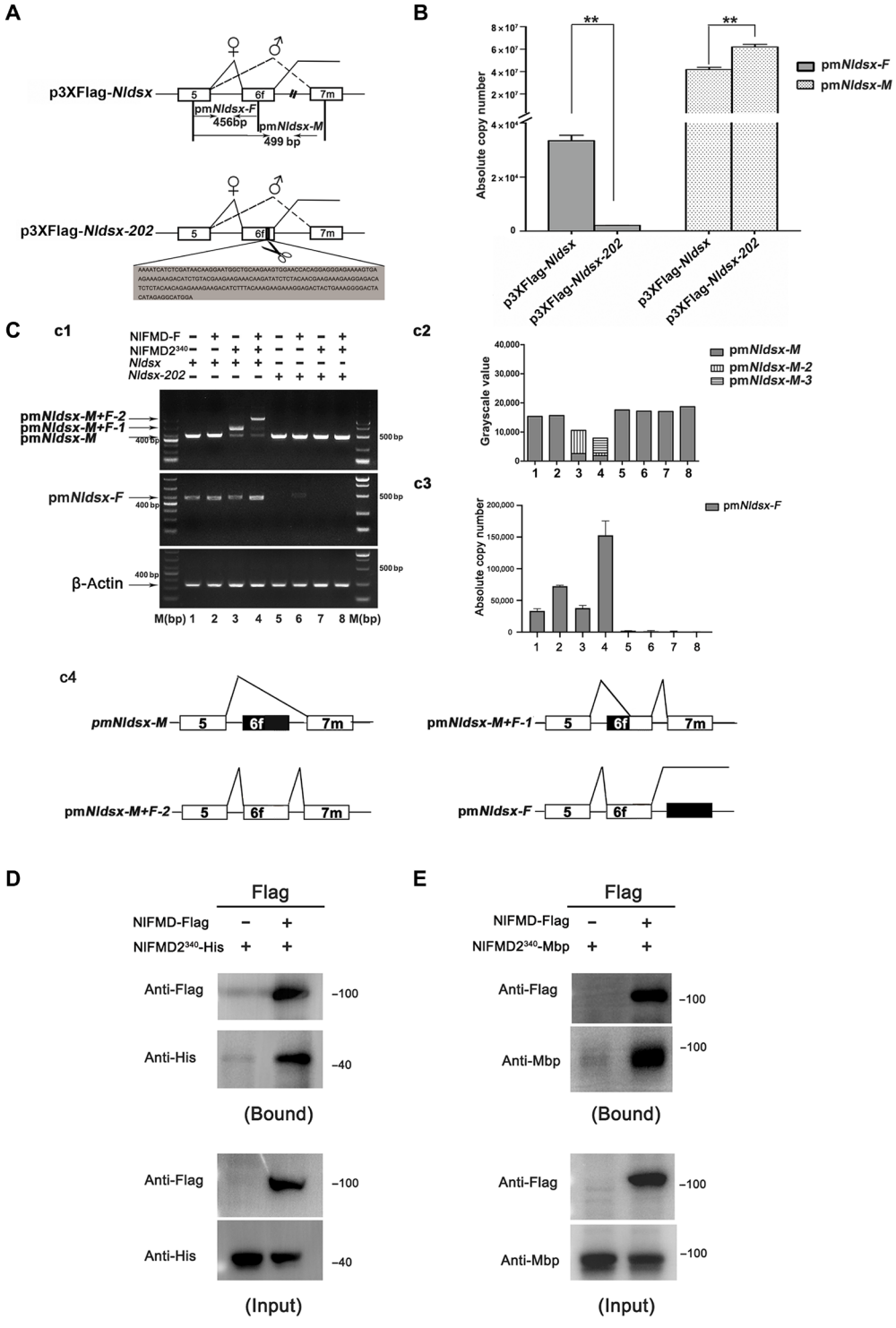


Fig. 5. Effect of *Nlfmd-F* and *Nlfmd2*^{340} products on the splicing patterns of *Nldsx* and *Nldsx-202* pre-mRNA in 293T cells. (**A**) Mini-genes and its default splicing in 293T cells. Boxes and the lines between boxes represent the exon and intron sequences, respectively. In the p3XFlag-*Nldsx-202*, 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 pm*Nldsx-F* and pm*Nldsx-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 pm*Nldsx-F* and pm*Nldsx-M* were used to test the splicing changes by semiquantitative RT-PCR. (c2) The grayscale value of the pm*Nldsx-M*, pm*Nldsx-M+F-1*, and pm*Nldsx-M+F-2* bands was measured by software Image J. (c3) The expressions of *Nldsx-F* were measured by absolute quantification with primer pm*Nldsx-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-F immunoprecipitated (IP) with *Nlfmd2*^{340}. (**E**) NIFMD-Flag pull-down assay with NIFMD2^{340}-Mbp. Data in (B) are represented as means ± SEM. Student's *t* test was used in (B) (**$P < 0.001$).

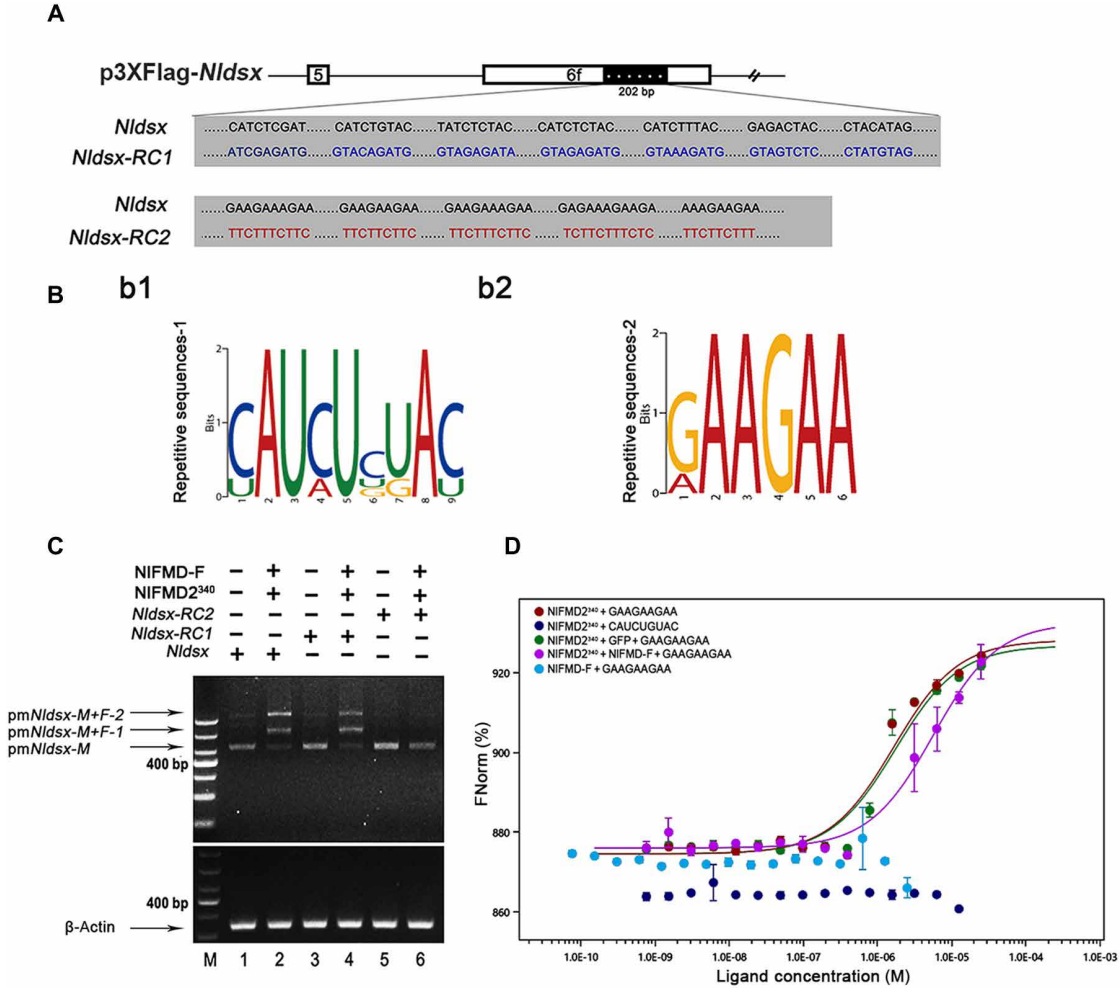


Fig. 6. Important elements involved in the regulation of *Nldsx* pre-mRNA alternative splicing. (**A**) Regulatory elements of *Nldsx* female-specific splicing (top) and mutant sequences RC1 and RC2 in which the repetitive regulatory elements were changed to their reverse sequences. (**B**) Repeat sequences that were identified using the MEME Suite. (**C**) Results of semiquantitative analysis with primer pm*Nldsx-M*. The results showed that *Nlfmd-F* and *Nlfmd2*^{340} regulated the splicing of p3XFlag-*Nldsx* and p3XFlag-*Nldsx-RC1*; *Nlfmd-F* and *Nlfmd2*^{340} had no influence on the splicing of p3XFlag-*Nldsx-RC2*, indicating that mutation in RC2 abolished the Nlfmd-F/Nlfmd2–mediated regulation of *Nldsx* splicing. (**D**) NIFMD-F/NIFMD2^{340} binding with GAAGAAGAA as demonstrated by microscale termophoresis (MST). Red indicates protein NIFMD2^{340} bound with repetitive sequences GAAGAAGAA with dissociation constant $K_D = 1.6366 \pm 0.49644$ μM, navy blue indicates NIFMD2^{340} could not bind with repetitive sequences CAUCUGUAC, green indicates NIFMD2^{340} and GFP bound with GAAGAAGAA with dissociation constant $K_D = 1.709 \pm 0.47464$ μM, pink indicates NIFMD2^{340} and NIFMD-F bound with GAAGAAGAA with dissociation constant $K_D = 5.5995 \pm 1.3$ μM, and blue indicates that NIFMD-F could bot bind with GAAGAAGAA. Both repetitive sequences were labeled by Cy3. Data in (D) are represented as means ± SEM.

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 NIFMD2^{340} and NIFMD-F may interact. In the vertebrate 293T cells, NIFMD2^{340} 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 NIFMD2^{340} to the RAAGAA repeat motif (*35*). In summary, we have presented evidence suggesting that *Nlfmd2* is involved in sex determination in BPH, and the SQUID-like protein NIFMD2^{340} 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 *Nlfmd-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

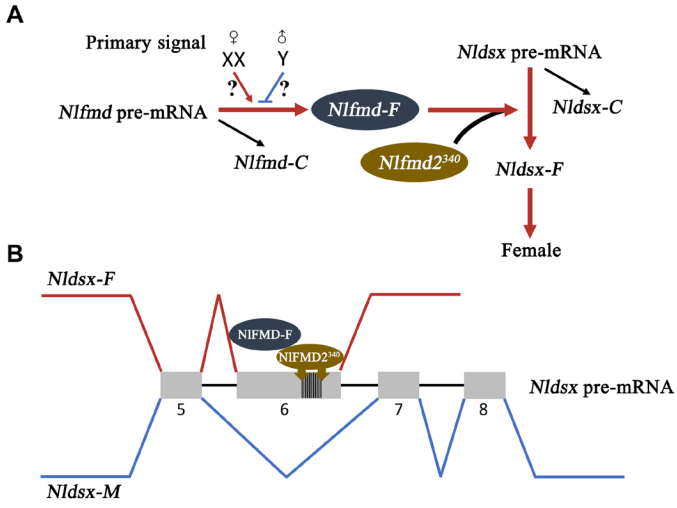


Fig. 7. A proposed model for sex determination in BPH. (**A**) Model for sex determination pathway of BPH. In female of BPHs, *dsx* alternative splicing process is controlled by three upstream genes, *NlFmd-F*, and *NlFmd2*. *NlFmd-F* needs the help of *NlFmd2* to maintain the female-specific *dsx-F* 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^{340} 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^{340} binds the RAAGAA repeat ESEs in the *Nldsx* 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^{340} 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 Mg^{2+} 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. 6110A). The internal control for quantitative RT-PCR (qRT-PCR) was the 18*S* rRNA gene of BPH, and the SYBR Premix ExTaq 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 (TIANGEN, 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 30 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-*Nl18s*, 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

$$\mathrm{DNA(copy)} = \frac{6.02 \times 10^{23}(\mathrm{copy/mol}) \times \mathrm{DNA\ amoumt(g)}}{\mathrm{DNA\ length(bp)} \times 660(\mathrm{g/mol/dp})} \ (49, 50) \quad (1)$$

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 *Nlfmd2*^{340} 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 (Takara, Dalian, China) following the manufacturer's instructions.

Third instar nymphs were injected with ds*gfp* or ds*Nlfmd*, 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) (Invitrogen, 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 Hepes, 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 MicroImaging, 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 ds*gfp* and ds*Nlfmd* and ds*Nlfmd2*^{340}, 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 *Nlfmd2*^{340} (GenBank accession number MW082037) and BPH genomes (GenBank accession number PRJNA177647) were used to search single guide RNAs (sgRNAs) used in CRISPR-Cas9 by sgRNAcas9 algorithm (*52*), and two sgRNAs with lowest off-target possibility were predicted to target *Nlfmd-F* and *Nlfmd2*^{340}, 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-SpCas9 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 G_0 were extracted and tested, and then the mutated males were mated with wild-type females to produce G_1. In G_1, the mutated males were mated with mutated females to produce G_2, and the number of males and females in G_2 was counted; some of them were used for genotyping the mutation, and the eggs of G_1 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 U/ml) at 37% in a humidified incubator that contained 5% CO_2. 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*, *Nlfmd2*^{340}, *Mini-Nldsx*, and the mutants were amplified by PCR and cloned into p3XFlag-CMV for the mini-*Nldsx* system; *Nlfmd2*^{340} 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 NlFMD2^{340} (50 μM, prokaryotic expression) was measured in PBS. The concentration of labeled RNA was constant at 20 nM; purified protein NlFMD2^{340} 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 K_D values. Data were analyzed using MO.Affinity Analysis software version 2.2.4.

Purified protein NlFMD-F (2.5 μM, Eukaryotic expression) and GFP (2.5 μM, prokaryotic expression) were added to NlFMD2^{340} (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 K_D values. Data were analyzed using MO.Affinity Analysis software version 2.2.4. The binding between NlFMD-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 Na_2HPO_4, and 1.4 mM KH_2PO_4 (pH 7.4)] and lysed with buffer [25 mM tris-HCl (pH 7.5), 150 mM MaCl, 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.org/doi/10.1126/sciadv.abf9237

View/request a protocol for this paper from *Bio-protocol*.

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