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A male-determining factor in the mosquito *Aedes aegypti*

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Sex determination in the mosquito *Aedes aegypti* is governed by a dominant male-determining factor (M factor) located within a Y chromosome–like region called the M locus. Here, we show that an M-locus gene, *Nix*, functions as an M factor in *A. aegypti*. *Nix* exhibits persistent M linkage and early embryonic expression, two characteristics required of an M factor. *Nix* knockout with CRISPR/Cas9 resulted in largely feminized genetic males and the production of female isoforms of two key regulators of sexual differentiation: *doublesex* and *fruitless*. Ectopic expression of *Nix* resulted in genetic females with nearly complete male genitalia. Thus, *Nix* is both required and sufficient to initiate male development. This study provides a foundation for mosquito control strategies that convert female mosquitoes into harmless males.

Insects employ diverse molecular mechanisms to determine sex (*1*–*3*). Sex is determined by X chromosome dosage in fruit flies (*4*), heterozygosity of the complementary sex determiner locus in honeybees (*5*), and by a female-specific piRNA in the silkworm *Bombyx mori* (*6*). Similar to mammals, sex determination in many insects is governed by an M factor located on a Y chromosome or homomorphic sex-determining chromosome (*1*). Despite the availability of genomic resources, no M factor has yet been characterized in any insect due to the difficulties of identifying genes in repeat-rich regions (*1*–*3*). Sex determination in mosquitoes is of particular interest as only adult females transmit pathogens responsible for dengue and yellow fever (*7*, *8*). Consequently, a mosquito M factor would be useful in implementing vector control strategies where female mosquitoes are converted into harmless males (*7*).

Male development in *A. aegypti* is initiated by an M factor located on the homomorphic sex-determining chromosome within a Y chromosome-like region called the M locus (*9*–*11*). The highly repetitive nature of the *A. aegypti* M locus has impeded the discovery of an M factor (*3*, *12*, *13*). To overcome this bottleneck, we developed the chromosome quotient (CQ) method to find male-specific (M-linked) genomic sequences by comparing the ratio of female to male alignments to reference sequences (*12*, *13*). First, we separately sequenced the genomes of males and females from two strains of *A. aegypti*: Liverpool and kh^w. Then, we generated a rudimentary assembly using the male kh^w strain data because repeat-rich regions like the M locus are often underrepresented in Sanger-based genome assemblies (*14*). Next, we aligned the male and female Illumina data to this assembly and identified 164 contigs that were potentially M-linked (defined as more than 5X as many alignments from male data as from female data in both strains) (table S1). Of the 164 sequences, 140 were either absent from RNAseq data altogether, absent from early embryo RNAseq samples, or present in female-derived RNAseq samples. Within the 24 remaining sequences, we only identified one new gene that is a distant homolog of *transformer-2* (table S2), which is involved in the splicing of *doublesex* (*dsx*) and *fruitless* (*fru*), two key regulators of sexual differentiation in *D. melanogaster* (*4*). We named this gene *Nix*. Because of the tantalizing link to sex determination, we hypothesized that *Nix* may function as an M factor in *A. aegypti*.

The *Nix* cDNA spans 985 bp and encodes a 288 amino acid polypeptide containing two RNA recognition motifs (GenBank KF732822) (fig. S1 and tables S2 and S3). Primers for *Nix* amplified a PCR product exclusively in male genomic DNA (Fig. 1A). We previously described two transgenes (J2 and sensor) that closely flank the M locus (*13*). Fluorescence in situ hybridization (FISH) to mitotic chromosomes using $M^{J2sensor}/m$ males confirmed that the *Nix* signal localizes to only one homologous copy of chromosome 1 at position 1q21, the location of the M locus (Fig. 1D and fig. S2) (*11*). Digital droplet PCR indicated that one haploid copy of *Nix* is present in males and zero copies of *Nix* are present in females (Fig. 1E). Next, we analyzed whether recombination could separate *Nix* from the M locus. By screening 5,000 individuals, we identified 19 recombinants where the J2 transgene was separated from the M locus (*13*, *15*). In females from a colony established from these individuals, we could not identify *Nix* by PCR, supporting the conclusion that *Nix* is located within the M lo-

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cus (Fig. 1B). Transcription of *Nix* was first detected 3-4 hours after oviposition (Fig. 1C and fig. S3), corresponding to the beginning of the syncytial blastoderm stage before sex is established (*16*). Thus, *Nix* exhibits two necessary characteristics of an M factor: persistent M linkage and early embryonic expression.

To investigate the role of *Nix* in mosquito development, we generated somatic loss-of-function mutants by injecting CRISPR/Cas9 (*17*, *18*) and synthetic guide RNAs (sgRNAs) targeting *Nix* into embryos oviposited by females that had mated with double-marked $M^{J2sensor}$/m transgenic males (fig. S4). In the absence of any phenotypic changes, virtually all males resulting from this cross would be double-marked, whereas all females would be unmarked. Genetic lesions in *Nix* were confirmed by RNAseq and DNA sequencing, and were associated specifically with *Nix* guide RNA target sites (fig. S5 and table S4).

Somatic knock-out of *Nix* resulted in feminization or deformities in sexually dimorphic organs in more than two-thirds (55/79) of double-marked males (designated hereafter as *nix*⁻ males) whereas unmarked females (control) were morphologically typical (Fig. 2A and figs. S6 to S8). As somatic mosaics, levels of feminization were variable among *nix*⁻ individuals. The phenotype of each *nix*⁻ male was scored for the extent of feminization (table S5 and figs. S6 to S8). A common morphological feminization that appeared in 53% (42/79) of *nix*⁻ males was the absence of one or both gonocoxites and gonostyli, features specific to male genitals used to grasp the female during mating (Fig. 2E and figs. S6 and S7) (*19*). We also observed feminized antennae with fewer and shorter setae than normal males in 44% (35/79) of *nix*⁻ males (Fig. 2D and figs. S6 and S8).

We further investigated the molecular mechanism of the feminization of *nix*⁻ males. *Dsx* and *fru* are essential genes in the sex determination pathway of many insects and differential splicing of each results in a downstream cascade that programs the development of sexually dimorphic traits (*20*–*23*). We confirmed that *nix*⁻ males produced female splice variants of both *dsx* and *fru* at 0.47x and 1.44x the amounts in wild-type females respectively (Fig. 2, B and C, table S6, and fig. S9). Using RNAseq to examine the expression of sex-biased genes in *nix*⁻ males, we found a global feminization of sex-biased gene expression consistent with the observed morphological feminization and the key regulatory functions of *dsx* and *fru* (Fig. 2, F and G, and fig. S10). Thus, *Nix* is required to initiate male development and functions upstream of the two master regulators of sexual differentiation.

To determine if *Nix* was sufficient for male determination, we investigated the effect of ectopic expression of *Nix* in genetic females. Embryos oviposited by females that had mated with double-marked $M/m^{J2sensor}$ transgenic males were injected with a plasmid expressing *Nix* under the control of the *A. aegypti polyubiquitin* promoter (fig. S4) (*24*). In this case, virtually all genetic females would be double-marked, whereas genetic males would be unmarked. In our first experiment, more than two-thirds (16/23) of the double-marked females (designated hereafter as *nix*⁺ females) exhibited extensive masculinization or deformities of the genitalia (Fig. 3A, table S7, and fig. S11). Two male-specific structures of the external genitalia, the gonocoxites and gonostyli (*19*), were clearly visible in 43% (10/23) of *nix*⁺ females whereas a further 26% (6/23) had deformed genitalia (Fig. 3, A and C, and table S7). Testes were identified in 34% (8/23) of *nix*⁺ females and accessory glands; vasa deferentia were identified in 60% (14/23) of *nix*⁺ females. (Fig. 3, B to D, table S7, and fig. S11). In a second experiment 27% (5/18) of *nix*⁺ females exhibited masculinized or deformed genitalia (Fig. 3A and table S7). Thus, *Nix* is sufficient to initiate male development.

Using Illumina sequences from male genomic DNA and male RNAseq, we identified a homolog of *Nix* in the Asian tiger mosquito, *A. albopictus*, with 52% identity at the amino acid level (e-value = 1e-71) (GenBank KP765684 and figs. S12 and S13). This gene is only found in male genomic DNA and is expressed in adult males and early embryo of *A. albopictus*, suggesting that *Nix* may be a conserved M factor in these *Aedes* mosquitoes. We also searched for *Nix* in other mosquito genera including *Culex* and *Anopheles* but only found autosomal or X-linked genes with RNA recognition motifs.

Here we demonstrate that an M-locus gene, *Nix*, is an M factor in *A. aegypti* because it is both required and sufficient to initiate male development, although complete sex conversion has not been achieved in our transient assays. *Nix* encodes a potential splicing factor and the absence of *Nix* shifts the alternative splicing of *dsx* and *fru* toward their female isoforms. The discovery of *Nix* provides an opportunity to characterize the remaining genes and interactions in the *A. aegypti* sex determination pathway, which may be informative in unraveling the sex determination cascades of mosquitoes in general.

Aedes aegypti is a major vector for dengue, yellow fever, and chikungunya viruses, and only female mosquitoes feed on blood and transmit these pathogens. Thus, genetic control methods that introduce a male bias to reduce mosquito populations are attractive and potentially effective measures to reduce the incidence of mosquito-borne disease (*7*, *8*). When dosage compensation and sex determination are linked, as in the silkworm, manipulation of the sex determination pathway results in sex-specific embryonic lethality due to misregulation of dosage compensation (*6*). In contrast, we have obtained partial sex-change phenotypes from both *Nix* knockout and ectopic expression, presumably because *A. aegypti* does not require dosage compensation. Thus, this study provides the foundation for developing mosquito control strategies by converting females into harmless males or selectively eliminating deadly females.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S14
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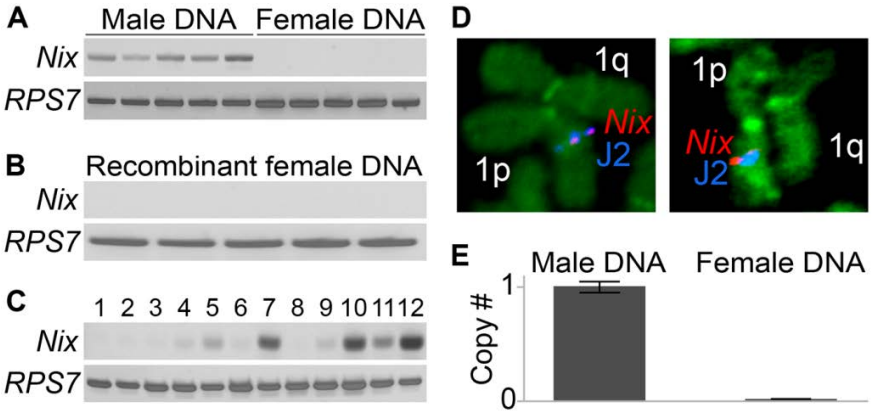


Fig. 1. *Nix* is located within the M locus. (**A**) PCR for *Nix* in male and female genomic DNA. For all PCR experiments, a ribosomal protein gene, *RPS7*, was used as a positive control. (**B**) PCR for *Nix* in genomic DNA from recombinant female $m^{J2sensor}$/m *A. aegypti*. (**C**) RT-PCR expression profile of *Nix* from 0-12 hour embryo cDNA starting at 0-1 hours in 1 hour increments. (**D**) FISH with a probe for *Nix* and the J2 transgene in mitotic chromosomes of J2 transgenic males. (**E**) *Nix* copy number as determined by digital droplet PCR (ddPCR) on male and female genomic DNA. Error bars represent SEM.

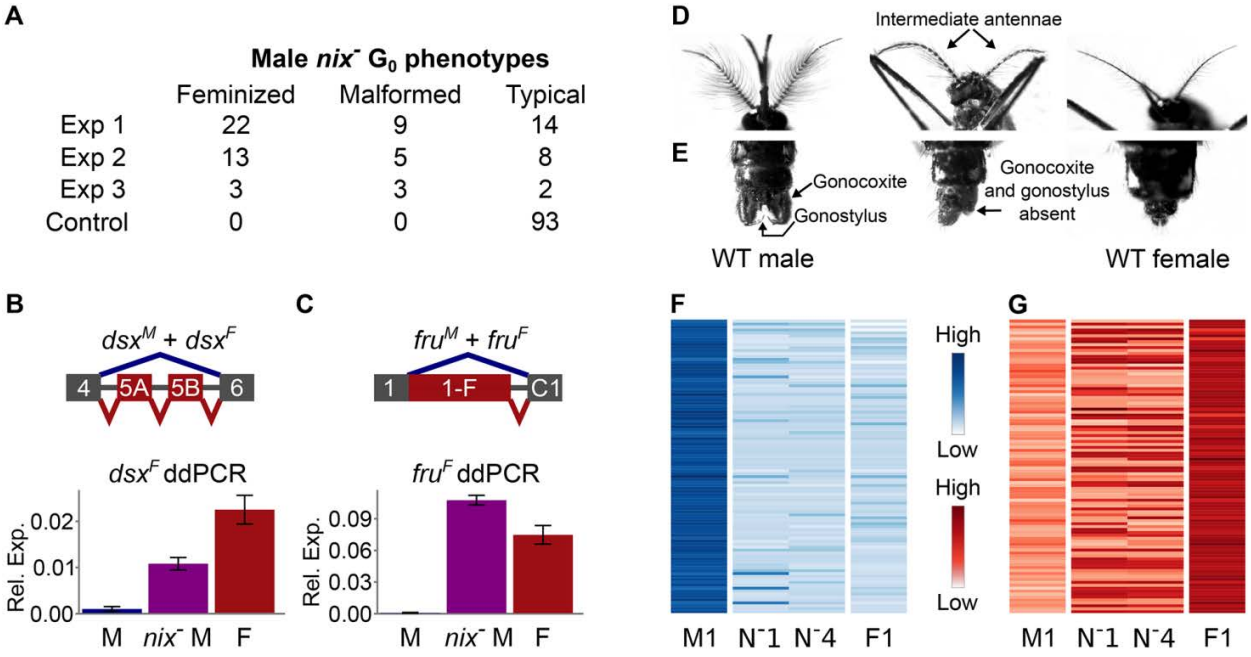


Fig. 2. Knockout with CRISPR/Cas9 demonstrates that *Nix* is required for male development. (**A**) Phenotypes of injected individuals. (**B**) The female isoform of *dsx* (dsx^F) is present in nix^- *A. aegypti* detected by ddPCR. Rel. Exp. – Relative expression. Error bars represent SEM. (**C**) The female isoform of *fru* (fru^F) is present in the nix^- *A. aegypti* detected by ddPCR. Error bars represent SEM. (**D**) Feminization of the antennae in a nix^- male individual. (**E**) Feminization in the genitals of a nix^- male individual. (**F** and **G**) The log2 RPKM expression level heat map of the top 100 male-biased (F) and female-biased (G) genes in wild-type males, nix^- male individuals, and wild-type females. Two heat maps from nix^- male are shown here. All other heat maps are shown in fig. S10.

A **Female *nix*⁺ G_0 external phenotypes**

	Masculinized	Malformed	Normal
Exp 1	10	6	6
Exp 2	2	3	13
Control	0	0	134

B **Female *nix*⁺ G_0 male features**

	Testes	Acessory glands	Claspers
Exp 1	8	14	9

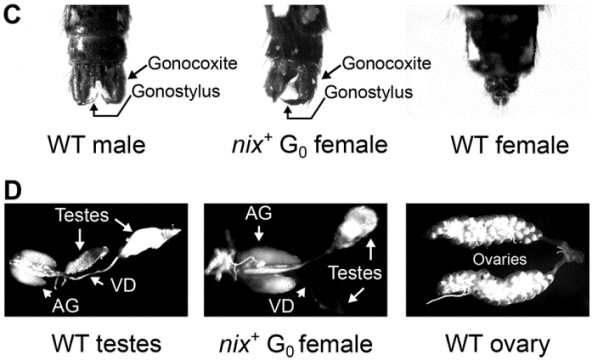


Fig. 3. Ectopic expression demonstrates that *Nix* is sufficient to initiate male development. (**A**) The phenotypes of *nix*⁺ females. Thirty individuals were sacrificed at the larval stage to examine gene expression and therefore have an undetermined phenotype. (**B**) The number of *nix*⁺ females with male-specific features from experiment 1. (**C**) Wild-type genitals compared to the genitals of a *nix*⁺ female which have gonocoxites and gonostyli. (**D**) Wild-type testes and ovaries compared to gonads of a *nix*⁺ female which had testes and accessory glands. Wild-type images and *nix*⁺ images are viewed under x55 and x80 magnification respectively. AG, accessory glands; VD, vas deferens.