SEX DETERMINATION

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 clustered regularly interspaced short palindromic repeats (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 a female-specific Piwi-interacting RNA 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 because 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 *Aedes 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 *Aedes aegypti* M locus has impeded the discovery of an M factor (*3*, *12*, *13*). To overcome this bottleneck, we developed the chromosome quotient 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 5 times as many alignments from male data as from female data in both strains) (table S1). Of the 164 sequences, 140 were either absent from RNA sequencing (RNA-seq) data altogether, absent from early embryo RNA-seq samples, or present in female-derived RNA-seq samples. Within the 24 remaining sequences, we identified only 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 *Drosophila 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 base pairs 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 polymerase chain reaction (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 5000 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 locus (Fig. 1B). Transcription of *Nix* was first detected 3 to 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.

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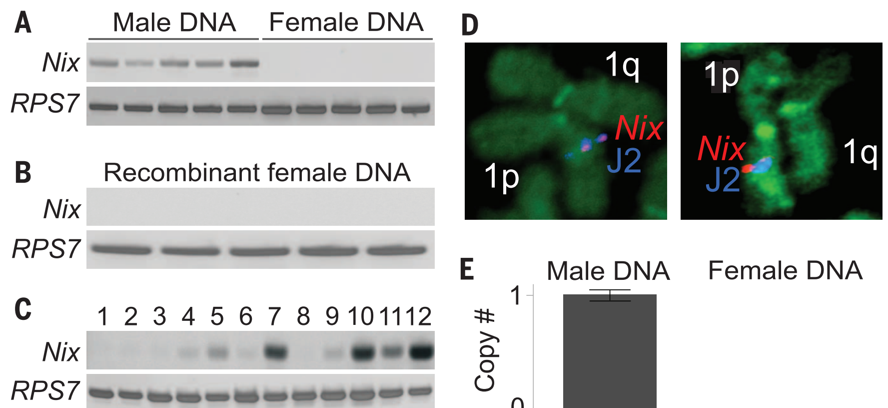
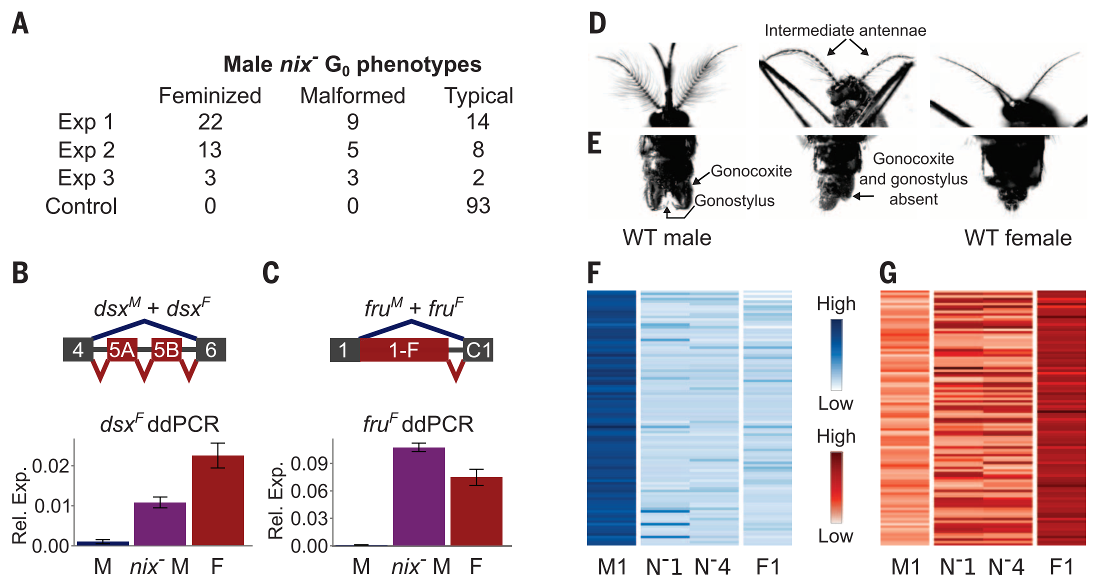


Fig. 1. *Nix* is located within the M locus. (**A**) PCR for *Nix* in male and female genomic DNA. For all PCR experiments, a ribosomal protein gene, *RPS7*, was used as a positive control. (**B**) PCR for *Nix* in genomic DNA from recombinant female m^{J2sensor}/m *A. aegypti*. (**C**) Reverse transcription PCR expression profile of *Nix* from 0- to 12-hour embryo cDNA starting at 0 to 1 hour 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, mean ± SEM.



Male nix^- G_0 phenotypes

	Feminized	Malformed	Typical
Exp 1	22	9	14
Exp 2	13	5	8
Exp 3	3	3	2
Control	0	0	93

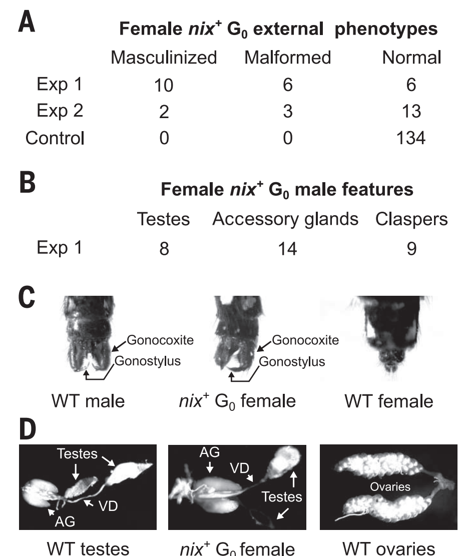
Fig. 2. Knockout with CRISPR-Cas9 demonstrates that *Nix* is required for male development. (**A**) Phenotypes of injected individuals. (**B**) The female isoform of *dsx* (dsx^F) is present in nix^- *A. aegypti* detected by ddPCR. Rel. Exp., relative expression. Error bars, mean ± SEM. (**C**) The female isoform of *fru* (fru^F) is present in the nix^- *A. aegypti* detected by ddPCR. Error bars, mean ± 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 reads per kilobase per million mapped reads expression level heat map of the top 100 male-biased (F) and female-biased (G) genes in wild-type males, nix^- males, and wild-type females. Two heat maps from nix^- males are shown here. All other heat maps are shown in fig. S10.

To investigate the role of *Nix* in mosquito development, we generated somatic loss-of-function mutants by injecting clustered regularly interspaced short palindromic repeats–Cas9 (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 RNA-seq and DNA sequencing and were associated specifically with *Nix* guide RNA target sites (fig. S5 and table S4).

Somatic knockout of *Nix* resulted in feminization or deformities in sexually dimorphic organs in more than two-thirds (55 of 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 of 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 of 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 0.47 and 1.44 times the amounts in wild-type females, respectively (Fig. 2, B and C; table S6; and fig. S9). Using RNA-seq 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 whether *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 of 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 of 23) of nix^+ females, whereas a further 26% (6 of 23) had deformed genitalia (Fig. 3, A and C, and table S7). Testes were identified in 34% (8 of 23) of nix^+ females and accessory glands; vasa deferentia were identified in 60% (14 of 23) of nix^+ females (Fig. 3, B to D; table S7; and fig. S11). In a second experiment, 27% (5 of 18) of nix^+ females exhibited masculinized or deformed genitalia (Fig. 3A and table S7). Thus, *Nix* is sufficient to initiate male development.



Female nix^+ G_0 external phenotypes

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

Female nix^+ G_0 male features

	Testes	Accessory 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 with the genitals of a nix^+ female, which have gonocoxites and gonostyli. (**D**) Wild-type testes and ovaries compared with gonads of a nix^+ female, which had testes and accessory glands. Wild-type images and nix^+ images are viewed under 55x and 80x magnification, respectively. AG, accessory glands; VD, vas deferens.

Using Illumina sequences from male genomic DNA and male RNA-seq, we identified a homolog of *Nix* in the Asian tiger mosquito, *A. albopictus*, with 52% identity at the amino acid level (e-value = 10^{-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 embryos 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 found only 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
Tables S1 to S9
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