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SEX DETERMINATION

A chimeric gene paternally instructs female sex determination in the haplodiploid wasp *Nasonia*

Yuan Zou[1], Elzemiek Geuverink[1], Leo W. Beukeboom[1], Eveline C. Verhulst[1,2,3]*, Louis van de Zande[1]*†

Various primary signals direct insect sex determination. In hymenopteran insects, the presence of a paternal genome is needed to initiate female development. When absent, uniparental haploid males develop. We molecularly and functionally identified the instructor sex-determination gene, *wasp overruler of masculinization* (*wom*), of the haplodiploid wasp *Nasonia vitripennis*. This gene contains a P53-like domain coding region and arose by gene duplication and genomic rearrangements. Maternal silencing of *wom* results in male development of haploid embryos. Upon fertilization, early zygotic transcription from the paternal *wom* allele is initiated, followed by a timely zygotic expression of *transformer* (*tra*), leading to female development. *Wom* is an instructor gene with a parent-of-origin effect in sex determination.

Insect sex determination is characterized by a large variation of primary instruction signals. In many species, this instruction is transduced by the transformer protein (TRA). Female-specific splicing of *tra* pre-mRNA yields a functional TRA protein (TRA-F), which splices *doublesex* (*dsx*) transcripts into a female-specific isoform, leading to female development (*1*–*4*). Absence of TRA-F results in male-specific splicing of *dsx* transcripts and male development. TRA-F also regulates *tra* transcript splicing into the female-specific isoform, thus establishing an autoregulatory loop of functional TRA-F production (*5*, *6*).

In many Diptera, dominant male-determining factors (*7*–*11*) instruct sex determination. In *Drosophila*, the dose of X-linked factors is the primary signal (*12*). Hymenoptera have haplodiploid sex determination: Haploid males develop from unfertilized eggs; diploid females develop from fertilized eggs. The only molecularly characterized instructor in Hymenoptera is *complementary sex determiner* (*csd*) of the honey bee (*13*–*15*), where female-specific splicing of transcripts from the *tra* ortholog, *feminizer* (*fem*), is needed for female development (*15*, *16*). The multi-allelic *csd* gene, a paralog of *fem*, contains a hypervariable region and instructs female development when heterozygous and male development when homozygous or hemizygous (*15*, *16*). However, most parasitoid wasps, including *Nasonia*, do not have *csd* (*17*), and the instructor signal in those haplodiploids is unknown.

For *Nasonia vitripennis*, a sex determination model was proposed in which a primary instructor gene is maternally silenced in unfertilized eggs, whereas fertilized eggs receive a nonsilenced paternal allele (*18*, *19*). This maternally silenced instructor gene is not *tra* itself, as in embryos from fertilized eggs both the

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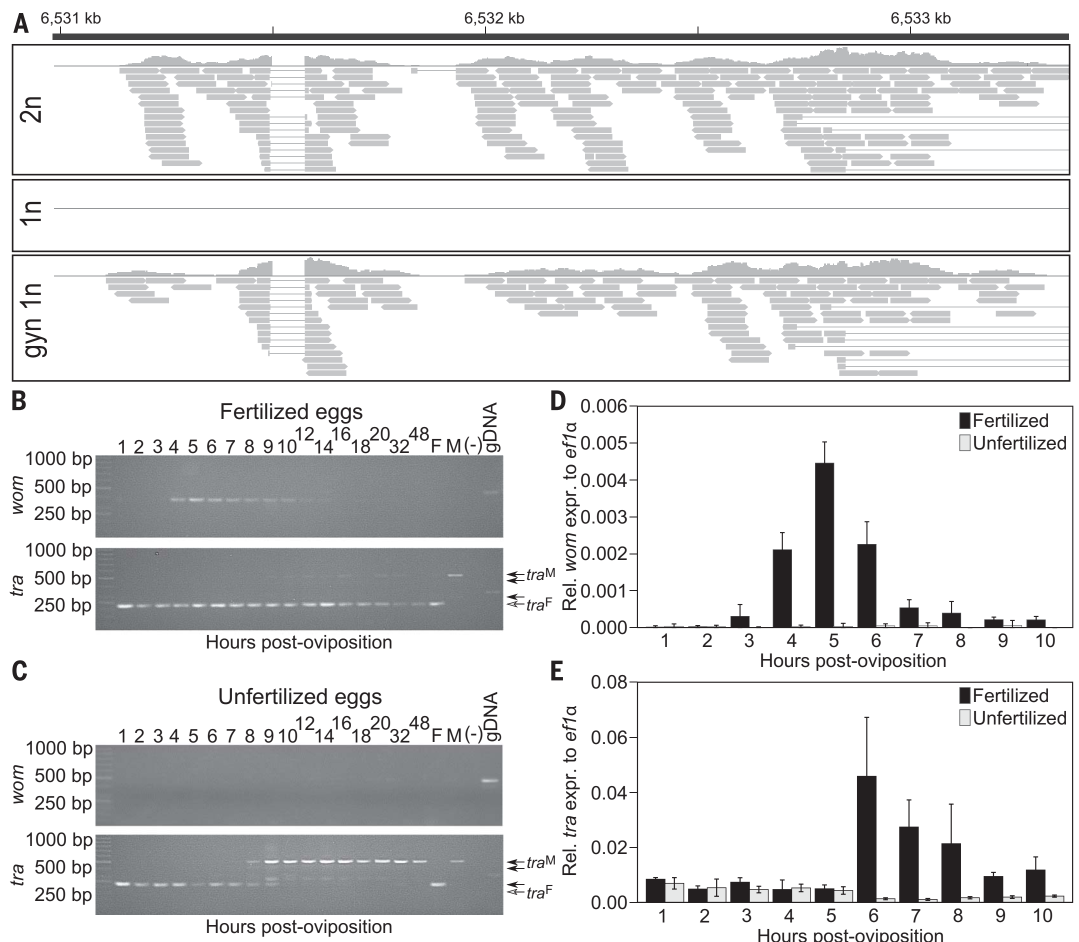


Fig. 1. Transcriptome analysis of early *Nasonia* embryos. (**A**) RNA-sequencing coverage (histograms) and read alignments (horizontal bars) from diploid female (2n, top), haploid male (1n, middle), and haploid gynandromorphic embryos (1n, bottom), located on chromosome 1, 6531.0-6533.5kb (NC_015867.2, reference *N. vitripennis* genome Nvit_2.1). (**B** and **C**) Temporal RT-PCR profiles of *wom* and *tra* for fertilized (B) and unfertilized eggs (C). Numbers indicate hours post-oviposition; F and M, adult female and male samples; (-), control without cDNA template; gDNA, adult male genomic DNA. Arrows indicate male-specific (filled) and female-specific (open) *tra* transcripts. (**D** and **E**). Temporal expression profiles of *wom* (D) and *tra* (E) for fertilized and unfertilized eggs. Reference gene: *EF1α*. Error bars: SEM.

maternal and paternal *tra* allele are expressed (*20*). Therefore, we suggested that an instructor gene is only expressed after fertilization in early diploid embryos and activates zygotic *tra* transcription (*21*, *22*). This initiates *tra* autoregulation, leading to female development.

To identify this regulatory gene, we sequenced transcriptomes of early embryos (see supplementary materials and methods for details). Haploid embryos (males) were obtained from unmated females; diploid embryos (females) were obtained from mated females, although their clutches contain ~20% unfertilized haploid eggs as well. Additionally, we used haploid embryos from unmated females of a gynandromorph strain (HiCD12) of which ~40% develop into adults with female characteristics (*23*).

Zygotic *tra* expression starts at 5 hours postoviposition (hpo) in the syncytial stage (*19*). Therefore, transcriptome analysis was performed on 2- to 5-hpo embryos. Differentially expressed gene (DEG) analysis yielded only two loci, *tra* and *LOC103317656* (reference *Nasonia vitripennis* genome Nvit_2.1, Annotation release 101), to be more highly expressed in diploid than in haploid embryos. Both loci were also among the more highly expressed genes in gynandromorphic embryos compared to normal haploid embryos (table S1). The differential expression of *tra* is in agreement with previous results and is an essential feature of *Nasonia* sex determination (*19*). Many reads from diploid and gynandromorphic embryos, but no reads from haploid embryos, mapped to the *LOC103317656* region (Fig. 1A). Therefore, *LOC103317656* on chromosome 1 is a compelling candidate for the regulatory gene, which we call *wasp overruler of masculinization* (*wom*).

Wom consists of three exons of 428, 268, and 1512 base pairs (bp), interrupted by two introns of 79 and 87 bp. It has a single splice form, and the coding sequence translates into a protein of 580 amino acids. The residues 91 to 285 form a P53-like DNA binding domain (fig. S1) that shares 30 to 43% identity with P53-family homologs and contains conserved zinc-binding, dimerization, and DNA binding motifs. These features correspond to the functional elements of P53 proteins for gene regulation and transcription in mammals (*24*–*26*). WOM also contains a coiled-coil domain at the C-terminal region (residues 550 to 577) (fig. S1), which may function in gene regulation (*27*, *28*).

Temporal expression analyses, with *tra* as internal control, revealed that *wom* is expressed in early diploid embryos but not in haploid embryos, demonstrating that it is not maternally provided (Fig. 1, B to D, and fig. S2A). Zygotic transcription of *wom* starts 2 to 3 hpo (blastoderm stage) and peaks at 4 to 5 hpo (Fig. 1D and fig. S2A), consistent with the DEG analysis. It coincides with the onset of zygotic *tra* expression that peaks at 6 to 7 hpo (*19*) (Fig. 1E and fig. S2B). *Wom* expression abruptly declines at 6 to 7 hpo (Fig. 1D and fig. S2A) and is not expressed during later female or male life stages (fig. S3). The sequential *wom* and *tra* expression within a defined time window is consistent with its proposed function of initiating zygotic *tra* transcription.

We determined the allelic origin of the *wom* transcripts using a synonymous single-nucleotide polymorphism (SNP) in exon 3 of *wom* (fig. S4A) that results in an Nhe I restriction fragment polymorphism. Sequencing and Nhe I digestion of reverse transcription–polymerase chain reaction (RT-PCR) products from the progeny of reciprocal crosses (Fig. 2A) demonstrated that *wom* mRNA is transcribed only from the paternal allele in diploid embryos (Fig. 2B and fig. S4B), in line with the hypothesis that *wom* is maternally silenced.

To confirm that *wom* is involved in initiating zygotic *tra* expression, we silenced *wom* in early diploid embryos by parental RNA interference (pRNAi) (*29*, *30*). This resulted in a reduction of both *wom* and *tra* expression levels in *wom* pRNAi diploid embryos, at 4 to 5 hpo for *wom* [P < 0.001; Welch's analysis of variance (ANOVA) with Tukey's test; Fig. 3A and fig. S5A] and 6 to 8 hpo for *tra* (P < 0.05; Welch's ANOVA with Tukey's test; Fig. 3B and fig. S5B). Furthermore, the diploid embryos had male-specific *tra* transcripts (fig. S5C) and developed as fully fertile males (Fig. 3C, fig. S6, and table S3). *Wom*-silenced females produced an increased number of haploid sons. This might be a derived *wom* silencing effect, as females can adjust the fertilization rate of their eggs. Conversely, *tra* pRNAi had no effect on the early embryonic *wom* expression in diploid embryos (P > 0.05; Welch's ANOVA with Tukey's test; Fig. 3A and fig. S5A), but it reduced *tra* expression levels (P < 0.05; Welch's ANOVA with Tukey's test; Fig. 3B and fig. S5B). This confirms that *wom* acts upstream of *tra* in the *N. vitripennis* sex-determining cascade and is essential for initializing zygotic *Nvtra* expression.

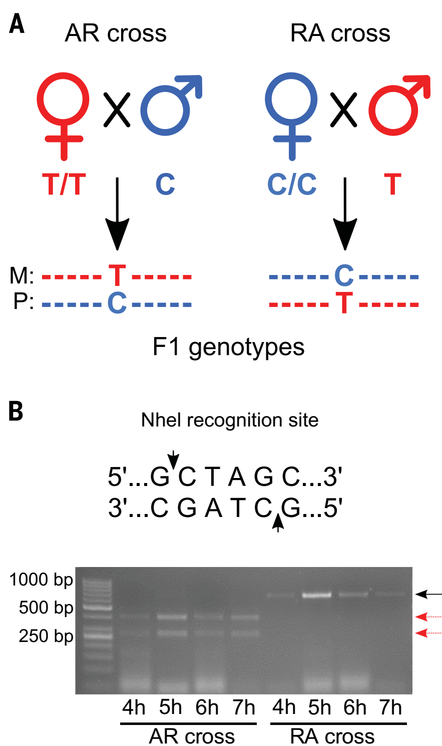
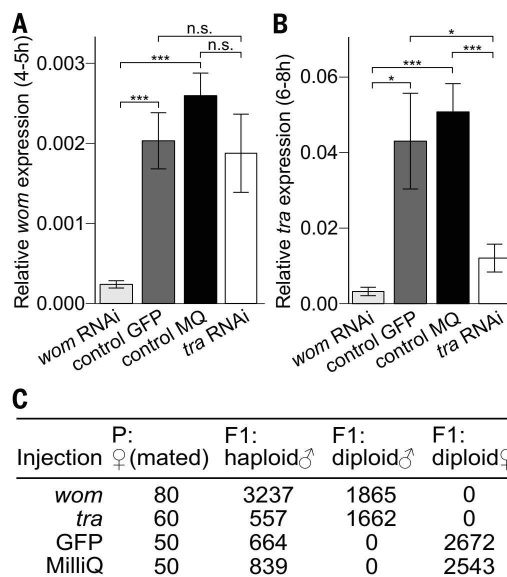


Fig. 2. *Wom* is expressed from the paternal allele only. (**A**) Crossing scheme to test the allelic origin of the *wom* transcripts. M, maternal; P, paternal. (**B**) Restriction fragment length polymorphism analysis of F_1 embryos 4 to 7 hpo. Arrows indicate undigested (black) and digested (red) PCR products.



Injection	P: ♀(mated)	F1: haploid♂	F1: diploid♂	F1: diploid♀
wom	80	3237	1865	0
tra	60	557	1662	0
GFP	50	664	0	2672
MilliQ	50	839	0	2543

Fig. 3. *Wom* and *tra* expression in early zygotes from dsRNA-treated females. *Wom* (**A**) and *tra* (**B**) expression in 4- to 5-hpo embryos of mated *wom* double-stranded RNA (dsRNA)– or *tra* dsRNA–injected females. Error bars depict SEM. *P ≤ 0.05, ***P ≤ 0.001; n.s., not significant. (**C**) Number of *wom* dsRNA– or *tra* dsRNA–injected females and their offspring number. GFP (green fluorescent protein)– and MilliQ-injected females served as controls.

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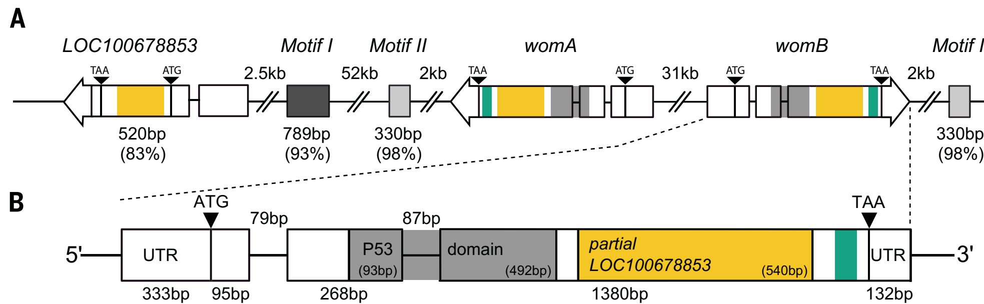

Fig. 4. Genomic organization of the *wom* locus. (**A**) Two copies, *womA* and *womB*, are present in antiparallel orientation. The medium gray block represents the p53-like domain. The downstream yellow block represents the partial *LOC100678853* sequence (yellow). P53-like domain sequence motifs I (dark gray box) and II (light gray box) are located between *womA* and *LOC100678853*. Motif II has an identical copy 2 kb downstream of *womB*. (**B**) Detailed structure of *womB*. Boxes represent exons, lines represent introns. The green block represents the coiled-coil region. UTR, untranslated region.

The genomic organization of *wom* is complex. The gene is duplicated as two antiparallel oriented copies (*womA* and *womB*) separated by an intergenic region of 31 kb (Fig. 4A). Both copies are transcribed (fig. S7, A to C). Downstream of the P53-like domain, a region of 540 bp (994 to 1533) shares 83% nucleotide identity with a 520-bp region of the annotated *Nasonia*-specific gene *LOC100678853* (reference *Nasonia vitripennis* genome Nvit_psr_1.1, Annotation release 104) (Fig. 4B). This is a gene of unknown function, 58.5 kb downstream of *womA*. The intergenic region between *womA* and *LOC100678853* contains two motifs interspersed by 52 kb (Fig. 4A). Motif I (789 bp) has 93% similarity and motif II (330 bp) 98% similarity with the P53-like domain of *wom* (Fig. 4A). The complete sequence of motif II is included in motif I. An identical copy of motif II is located 2 kb downstream of *womB* (Fig. 4A). Apparently, this region of chromosome 1 has been a site of dynamic genomic rearrangements.

Wom and *LOC100678853* homologs were only detected in *Nasonia* species and their close relative *Trichomalopsis* (fig. S8). By contrast, *wom*-like genes lacking the partial *LOC100678853* homologous region were only found in three other pteromalids (*Cecidostiba fungosa*, *Cecidostiba semifascia*, and *Pteromalus puparum*) (fig. S8). These species also lacked the entire *LOC100678853* gene. Sequence alignment and comparison of nonsynonymous to synonymous substitutions suggest that *wom* is an evolutionary new, chimeric, gene (supplementary text and table S5).

The molecular identification of primary sex determination genes has proved to be challenging (*7*–*11*). The existence of a female instructor gene in *Nasonia* was predicted (*19*, *20*), but its identity remained unknown. Here, we demonstrate that *wom* is a de novo evolved instructor gene with a parent-of-origin effect. It is maternally silenced, but is transcribed from the paternal allele in fertilized eggs, to initiate female development.

The existence of *wom* also may be relevant in explaining two cases of uniparental female offspring in *Nasonia* (*23*, *31*). *Wom* is not completely maternally silenced in haploid HiCD12 early embryos (fig. S9A) that partially develop as females (*23*). It is unlikely that this is due to a *wom* mutation, as no potential functional differences were detected in the HiCD12 gene sequence or its 2.5-kb upstream region (fig. S10). We hypothesize that the putative *gynandromorph* (*gyn*) gene, which maps to chromosome 4, is involved in maternal silencing of *wom*, and a loss-of-function mutation in HiCD12 leads to (partial) failure of imprinting.

Uniparental female offspring have been observed in a polyploid line (*32*). Triploid females occasionally produce diploid female offspring without fertilization (*31*). This may be due to dosage disruption in polyploid females, possibly combined with a *wom* mutation, resulting in defective *wom* imprinting.

Requirement of the paternal genome for female development is characteristic for haplodiploid reproduction. *Wom* is identified here as an instructor gene for female development with a parent-of-origin effect. Like *csd* in the honey bee, it is located in a dynamic genomic region (*16*, *33*). This suggests that, within Hymenoptera, various genes have evolved upstream of *tra* to enable paternally mediated onset of female development.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Supplementary Text
Figs. S1 to S10
Tables S1 to S5
References (*34*–*52*)

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Paternal factor specifies female wasps

Not all animals have specialized sex chromosomes to determine their sex. In hymenopteran insects, for example, unfertilized eggs become males and fertilized eggs become females. Prior work showed that the paternal genome provides instruction for female development. Zou *et al.* identified a sex determination instructor gene, *wasp overruler of masculinization* , with parent-of-origin effect, in the parasitoid *Nasonia vitripennis*. It is only transcribed from the paternally provided genome in fertilized eggs to initiate female development. This discovery provides insights into the molecular basis and evolution of sex determination.

Science, this issue p. 1115

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