HHS Public Access

Author manuscript

Trends Parasitol. Author manuscript; available in PMC 2017 March 01.

Published in final edited form as:

Trends Parasitol. 2016 March ; 32(3): 219–229. doi:10.1016/j.pt.2015.12.003.

Control of mosquito-borne infectious diseases: Sex and gene drive

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Abstract

Sterile male releases have successfully reduced local populations of the dengue vector, *Aedes aegypti*, but challenges remain in scale and in separating sexes prior to release. The recent discovery of the first mosquito male determining factor (M factor), will facilitate our understanding of the genetic programs that initiate sexual development in mosquitoes. Manipulation of the M-factor and possible intermediary factors may result in female-to-male conversion or female killing, enabling efficient sex-separation and effective reduction of target mosquito populations. Given recent breakthroughs in the development of CRISPR-Cas9 reagents as a source of gene drive, more advanced technologies at driving maleness, the ultimate disease refractory phenotype, become possible and may represent efficient and self-limiting methods to control mosquito populations.

Why sex matters

Anopheles mosquitoes are the primary vectors of malaria, one of the most deadly and costly diseases in human history. *Aedes aegypti* is a major vector for dengue, yellow fever, and chikungunya viruses. Over the past 40 years, the increase in the burden of dengue disease has been driven by many factors, primarily urbanization, globalization, and an inability to effectively control *Ae. aegypti* breeding [1]. More recently, similar factors have led to the large scale emergence of viruses such as chikungunya [2] and Zika [3], both also vectored by *Ae. aegypti*. Current prevention of these mosquito-borne infectious diseases depends heavily on effective vector control (http://www.who.int/mediacentre/factsheets/fs117/en/), which is hindered by increasing insecticide-resistance.

Genetic strategies to control dengue based on the release of sterile, transgenic individuals are currently underway and have been successful where attempted [4, 5]. These strategies provide effective mosquito control only as long as releases continue, and thus represent a

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long-term financial and administrative commitment that must be maintained even in the absence of continued transmission. For this reason, gene drive systems that either suppress a target population or permanently convert the target population into a refractory state by spreading effector genes have long been sought after [6-10], as both the release scale, duration and resistance to immigration pressure are expected to be dramatically lower than for the conventional sterile insect technique. Gene drive refers simply to the ability of a gene to be inherited more frequently than Mendelian genetics would dictate, thus, increasing in frequency, perhaps even to fixation [9]. As only females mosquitoes bite and transmit disease-causing agents, a desirable feature of both conventional sterile insect releases and gene drive strategies is that only male mosquitoes be released so that there is no potential for an increase in disease transmission. If females were released, they could also compete with targeted females in the field for mating with the released males. Therefore, females should be removed prior to release, and we use “sex-separation” to refer to this process in general. Novel methods to perform sex-separation or to otherwise eliminate disease-transmitting female mosquitoes would benefit tremendously from an understanding of the genetic basis for sex determination in mosquitoes.

Sex in insects: Diversity and model species

Sex is critical to the survival and evolution of sexually reproducing organisms. Among different insect species, diverse chromosome systems are employed to determine the sex of an individual. Such diversity can manifest within the same order, family, or even more closely related taxonomic groups. In the order Diptera, for example, sex-determining chromosome systems encompass a wide spectrum including XX/XY in the *Anopheles* mosquitoes and in *Drosophila* fruit flies, ZW/ZZ and XX/XO in some Tephritidae flies, and the homomorphic sex-determining chromosomes in *Culicinae* mosquitoes [11, 12]. Therefore, it is not surprising that the primary molecular signals or the ‘master switches’ of sex determination are also highly divergent and rapidly evolving in insects (e.g., [13]). Heterozygosity of the *complementary sex determiner* (*csd*) gene initiates female development in honeybees [14]. A recently discovered W chromosome-linked piRNA gene has been suggested as the female-determining factor in the silkworms [15]. In *Drosophila melanogaster* sex is determined by the dose of X-linked signal elements that encode transcription factors [16]. The presence of two X chromosomes in female *D. melanogaster* triggers the expression of sex-lethal (*sxl*) from its early embryonic promoter, producing the early SXL protein (SXL_E). SXL_E initiates a cascade of sex-specific splicing events that produce the female isoforms of two key transcription factors, *doublesex* (*dsx*) and *fruitless* (*fru*) (Figure 1A). These female-specific DSX and FRU protein isoforms program female differentiation [11]. In male *D. melanogaster*, the single X chromosome is unable to trigger early *sxl* expression, leading to male-specific DSX and FRU protein isoforms which program male differentiation. The sex-specific splicing of *dsx* and *fru* pre-mRNA is controlled by a protein complex that includes *transformer* (TRA) and *transformer 2* (TRA2), where TRA confers sex-specificity (Figure 1A). In *D. melanogaster*, sex-specific splicing of the *tra* pre-mRNA is regulated by yet another splicing factor, the late embryonic SXL (SXL_L). Late embryonic *sxl* transcripts requires the aforementioned early SXL_E, which is

only produced in females, to achieve proper splicing to encode a functional SXL_L protein (Figure 1A).

Sex in mosquitoes: Discovery of a male determining factor (M factor)

Previous genetic evidence indicates that sex determination in mosquitoes is controlled by a dominant M factor [11]. The M factor resides on the heteromorphic Y chromosome in *Anopheles* mosquitoes and in the M-locus of the homomorphic sex-determining chromosome in *Culicinae* mosquitoes, including species in the *Aedes* and *Culex* genera [17, 18]. In one key study, *An. culicifacies* of the XXY genotype was found to be male [19], suggesting the existence of a dominant M factor on the Y chromosome. This is similar to humans and a number of non-*Drosophila* flies but in contrast to *D. melanogaster*, where no dominant M factor exists and an XXY individual develops as a female due to the presence of two X chromosomes [11]. Despite the apparent rapid evolution of the primary signals that determine sex, the downstream genes of the sex-determination pathway, *dsx* and *fru*, are highly conserved; sex-specific or sex-biased splicing of *dsx* and *fru* are widely reported in many insects, indicating conserved functions in programming sexual differentiation [20]. Sex-specific splicing of *dsx* has been described in both *Aedes* and *Anopheles* suggesting the presence of a TRA-like activity [21-23]. Conserved cis-regulatory elements that likely correspond to TRA/TRA2 binding sites are found in *An. gambiae* and *Ae. aegypti dsx* and *fru* genes [22-25]. Although a *tra* gene has yet to be found in any mosquito, it remains possible that the M-factor and perhaps additional factors may directly or indirectly inhibit the TRA/TRA2 splicing machinery to confer male-specific *dsx* splicing (Figure 1B) [24].

The genomic regions that harbor the M factor, namely the M-locus or the Y chromosome, may be considered genomic ‘black holes’ because they are full of repeats, are difficult to sequence and assemble, and are often absent from genome assemblies. An M factor had not been characterized in any mosquito or any insect until this year [25], despite the availability of extensive genomic resources and intense interest for basic research and potential mosquito control strategies. Using a newly developed bioinformatics algorithm that overcomes the aforementioned difficulties, we discovered a single novel gene named *Nix* in the M-locus of the yellow fever mosquito, *Ae. aegypti*. *Nix* is located within the M-locus, as confirmed via tight genetic linkage, physical mapping, and male-specific haploidy. *Nix* encodes a putative RNA-binding protein and is transcribed during the maternal-to-zygotic transition, prior to when sex is determined. When *Nix* was knocked-out with clustered regulatory interspaced palindromic repeat (CRISPR)-Cas9, genetic males were feminized, showing female genitals and antenna. When *Nix* was ectopically expressed, genetic females developed external and internal male genitalia including testis and accessory glands. Thus *Nix* is both required and sufficient to initiate male development and it functions as an M factor in *Ae. aegypti*. *Nix* knockout in feminized males produced the female splice variants of *dsx* and *fru*, and showed genome-wide feminization of gene expression consistent with the observed morphological feminization. Thus, *Nix* functions upstream of *dsx* and *fru*, two key regulators of sexual differentiation. Based on the predicted RNA-binding property of the NIX protein and its distant similarity to TRA2 [25], it may be hypothesized that NIX is a splicing factor. *Ae. aegypti dsx* has two female-specific exons designated exons 5a and 5b. Sequence analysis suggests that the 3’ splice site proceeding exon 5a is likely a preferred

splice site while the 3' splice site proceeding exon 5b is weak [22, 23]. The use of aforementioned weak 5b splice site in females may be promoted by a TRA/TRA2-type complex (Figure 1B). In males NIX may directly or indirectly inhibit TRA/TRA2 as well as the aforementioned 5a strong splice site, resulting in skipping of both exons 5a and 5b (Figure 1B). However, without biochemical evidence for the function of the predicted cis-regulatory sites, we cannot rule out the possibility that the female-specific splicing (e.g., the use of both 5a and 5b 3' splice sites in *dsx*) is the default. Under this scenario, NIX could inhibit the female-specific splicing in the males and a TRA-like activity may not be needed. A NIX homolog has been identified in *Ae. albopictus*, which showed male-specificity and embryonic expression. Although both species are in the *Stegomyia* subgenus, the estimated divergence time between *Ae. aegypti* and *Ae. albopictus* is ~70 million years [26]. Thus it will be interesting to determine the distribution and possible functional conservation of *Nix* in *Stegomyia* and other more divergent Culicinae species.

No *Nix* or any RNA-binding protein genes were found on the Y chromosome of *Anopheles gambiae* and *An. stephensi* despite the fact that extensive male Illumina sequences were available [27, 28]. However, a small number of protein-coding genes have been found in the aforementioned species using the same method that led to the discovery of *Nix* in *Ae. aegypti*. Of all the Y genes in *An. stephensi*, *Guy1* is the earliest to be transcribed [27, 28], at the very onset of maternal-to-zygotic transition. The timing of *Guy1* transcription and the fact that its transcription does not require any other factors from the Y chromosome suggest that it could function as an initiation or primary signal [27]. *Guy1* encodes a small protein, 56 amino acids in length, with predicted DNA-binding properties. In *An. gambiae*, gYG2 is the earliest Y gene to be transcribed and its transcription is also at the very onset of maternal-to-zygotic transition. It is not yet clear whether *Guy1* and *gYG2* are the M factors in these *Anopheles* mosquitoes. The Y chromosome is often sparse in genes while the X retains hundreds of genes. In *D. melanogaster*, a mechanism of complete dosage compensation exists to hyper-express the entire X chromosome in males in order to compensate for having only one copy of the X chromosome [29]. Although dosage compensation is not expected for insects with homomorphic sex-determining chromosomes including *Aedes* and *Culex* mosquitoes, complete dosage compensation has been clearly demonstrated in *Anopheles* [30]. Further complicating matters, *sxl*, *Fem/Masc*, and *xo-lethal 1* all regulate dosage compensation in addition to functioning as the master regulator of sex-determination in their respective organisms (*D. melanogaster*, *Bombyx mori*, and *Caenorhabditis elegans*) [15, 31, 32]. Thus, loss of function of *sex-lethal* or knockdown of *Masc* result in female embryonic lethality, not sex conversion, likely due to mis-regulation of dosage compensation [15, 33, 34]. It is not clear whether the M factor in *Anopheles* mosquitoes also regulates dosage compensation, but as candidate M factor genes are evaluated this is likely to be a complication.

Sex as a target for vector control

The sex-determination pathway can provide novel targets for generating genetic sexing strains [35] for sex-separation and for interference of the sex ratio to reduce mosquito populations. For example, knocking down the female isoform of *dsx* by RNAi in the larval stage resulted in female lethality [36]; alternative RNAi attempts resulted in reduced female

fecundity [37]. If full sex-conversion is to be achieved, it will at a minimum require manipulating both *dsx* and *fru*. Approaches that ectopically express dominant acting factors such as *Nix* or knock down a *transformer*-like intermediate upstream of *dsx* and *fru* may prove more efficient and effective. Despite extensive searches, *Nix* is the only gene found to be persistently linked to the M-locus in *Ae. aegypti* [25] and no unique small RNAs have been uncovered that are attributed to any M-linked sequences. Therefore, integration of *Nix* into the genome as a transgene may be sufficient to convert females into fertile males. Alternatively, *Nix* expression in the females may convert females into sterile males or simply confer female lethality (Figure 1C). All three outcomes could be readily exploited for sex-separation and population reduction. Tetracycline-regulated conditional expression [4, 5] may be used to maintain homozygous transgenic lines by suppressing *Nix* expression in females in the presence of tetracycline. Current high-profile trials using transgenic sterile mosquitoes require mechanical and manual separation of males and females [4, 5]. Converting female to fertile males would double the factory-scale male production and provide much more efficient population reduction than classic sterile insect techniques because of the added benefit of male-bias in subsequent generations [38]. Such increased male-bias over multiple generations will be even more profound when combined with the concept of gene drive (Figures 1C and 2). In essence, *Nix* could confer ‘maleness’, which may be considered the ultimate disease-refractory trait.

Gene drive: Is CRISPR/Cas9 different?

The concept of driving genes into wild populations to control vector-borne diseases is not new [6], and has been reviewed extensively over the past few decades [7-10]. More recently, such strategies have caught the attention of other disciplines [39-41]. However, engineering or harnessing chromosomal translocations [6], meiotic drive systems [42], transposable elements [43, 44], maternal-effect dominant embryonic arrest [45, 46], engineered underdominance [47, 48] and homing endonuclease genes (HEGs) [49] to achieve the goals of a gene drive-based vector control campaign have been slowed or prevented by the technical challenges associated with these systems, though many of these still hold great promise. Most recently, the advent of site-specific gene editing using CRISPR/Cas9 reagents has produced a wave of successful gene drive experiments in yeast [50], flies [51] and mosquitoes [52]. What sets CRISPR-based gene drive systems apart is not their novelty—they are a logical extension of past work—but their ease of production. Ultimately, the process of gene drive using CRISPR-based reagents is not substantially different than that using homing endonucleases or transposable elements (TE) (Figure 2). All three rely on the same homology-dependent repair processes encoded by the host organism following double-stranded DNA break (DSB) induction to increase their copy number (Box 1). One of the key ways these systems differ is simply in how the triggering DSB is induced. For TEs, the DSB is induced through excision of an integrated element (Figure 2A), whereas for HEGs and CRISPR the target site is specified by the nuclease (Figure 2B).

Observations that old laboratory colonies of *Drosophila* lacked the *P* transposable element, whereas all wild strains contained it suggested that this element had recently invaded flies around the globe [53, 54]. Subsequent work showed that *P* was capable of driving itself [55] and an additional cargo gene [56] through caged populations of *Drosophila;* results that

immediately caught the attention of the vector biology community [43]. While the *P* element is not active outside of Drosophilids [57] and other DNA elements such as *mariner/Mos*1 [58], Hermes [59], and *piggyBac* [60, 61] could not be efficiently remobilized in *Ae. aegypti*, highly efficient re-mobilization of *piggyBac* has been demonstrated in *An. stephensi* [62, 63]. Thus, a TE-based gene drive system using *piggyBac* may yet be feasible; this is especially intriguing considering *An. stephensi* have already been made resistant to the major human malaria parasite, *Plasmodium falciparum* [64].

Unlike TEs, which integrate relatively randomly in the genome, HEGs make a targeted DSB on the homologous chromosome when present in a hemizygous state. If the host cell machinery uses the HEG-containing chromosome as a template to repair the DSB, the HEG will increase in copy number (Figure 2B). Burt [49] theorized that this mechanism could be harnessed for gene drive in place of transposable elements, whose location in the genome could not be controlled. Following successful demonstrations that some HEGs can edit the genomes of *Anopheles* mosquitoes [65, 66], a HEG (I-*Sce*I) was found to be capable of driving itself into a cage population of *An. gambiae* when its target site was engineered in the homologous position [67]. Similar results were obtained in *Drosophila*, although the rate of homing was lower [68]. Another HEG, I-*Ppo*I, targets a highly conserved region of ribosomal DNA (rDNA). The fortuitous localization of rDNA repeats exclusively on the *An. gambiae* X chromosome has permitted the development of a HEG-based sex distortion system, where X-bearing sperm are killed yielding extremely male-biased progeny [69]. Combined with the ability to insert transgenes specifically on the Y-chromosome [70], a HEG-based gene drive system that converts a target population into all males now appears feasible for *An. gambiae*. The rDNA repeats are not X-specific in *Ae. aegypti* or most *Anopheles* mosquitoes, but our recent discovery of a male-determining gene in *Ae. aegypti* [25] opens up similar possibilities in engineering male-driving nucleases for this disease vector. A powerful feature of HEGs is their extreme specificity- a boon when considering potential off-target effects but a curse when trying to engineer a nuclease to recognize a new on-target site. Thus, efforts have been made to explore the potential of other site-specific nucleases to exhibit the same type of gene drive. Transcription-activator like element nucleases (TALENs) were engineered into a cage population of *Drosophila*, but the highly repetitive nature of these genes made them poor substrates for accurate DNA repair [71]. Even before the first functional data in mosquitoes were published, CRISPR was seen as an alternative programmable nuclease that could replace HEGs in gene drive strategies [40], with the first CRISPR-based gene drive published not long after in *Drosophila* [51].

Gene editing and drive with CRISPR/Cas9 in mosquitoes

The rapid development of CRISPR-based editing reagents [72-74] introduced a new programmable nuclease that does not suffer from the problems of HEGs (difficult to engineer) or TALENs (poor repair substrates). Gantz et al [52] recently described the successful CRISPR/Cas9-based drive of a 17 Kbp synthetic construct in the Asian malaria vector *An. stephensi*. For several generations, males containing the gene drive construct produced almost all transgenic offspring, defying Mendelian segregation. In their experiments, the Cas9 nuclease was active in the female germline as well, though somatic activity in the early embryos resulted in excessive mutations through non-homologous end-

joining [52]. While the effectiveness and long term stability of CRISPR-based gene drive constructs in mosquitoes remains unknown, these results are highly encouraging. We [25, 75] and others [76, 77] have demonstrated that CRISPR-based editing is highly effective in *Ae. aegypti* as well, where mutant phenotypes can be detected in injection survivors and characterized as somatic mosaics [25, 76], or used to generate heritable mutations [75]. Using a donor template, short oligonucleotide fragments or full gene cassettes can be incorporated into pre-specified locations in the *Ae. aegypti* or *Anopheles* genome at practical rates [52, 70, 75, 76], the first required step in a CRISPR-gene drive scenario. The power of the CRISPR system derives from the fact that nuclease (typically Cas9 protein) specificity can be determined via a single synthetic non-coding RNA less than 100 nt in length. The ease with which CRISPR nucleases can be generated, combined with the highly effective nature of CRISPR-based gene drive in *Drosophila* [51] has led to calls for increased regulatory capacity [41, 78] and institutional oversight [79] of CRISPR-based gene drive approaches, with some even calling for the prohibition of public discussion of the details due to fears of bioterrorism [80]. Esvelt [40] conceived of a “reversal” drive that could potentially restore a disabled gene to its original state in the case of adverse effects, or an “immunization” drive that could prevent the invasion of a maliciously introduced gene drive construct. Now experimentally validated in yeast [50], the former is better termed a “rescue” drive as true reversal does not actually occur; for both “reversal” and “immunization” drives concepts, functional Cas9 nuclease would remain in the target population in perpetuity [40], and could serve to catalyze the drive of additional sgRNAs introduced into the target population (Figure 2C). Detecting such a second wave of short guide RNAs in a Cas9-containing population could prove exceptionally difficult, given that the binding interactions between guide RNA and Cas9 protein are mediated primarily by the overall structure of the guide, with just a few critical base-specific contacts [81]. In essence, CRISPR-based gene drive systems are quite the opposite of HEGs. For HEGs, nuclease activity is intrinsically coupled with DNA target recognition-this is why they are so difficult to re-engineer. In contrast, for CRISPR systems, DNA target recognition (guide RNAs) and nuclease activity (Cas9) are completely distinct, and how to ensure continued specificity of a CRISPR-based gene drive construct once designed is a special challenge that remains to be resolved. The use of reversal/rescue drives would also not be applicable to gene drive occurring in an intergenic location (as there is no disrupted gene to rescue), such as may be preferred to drive pathogen-resistance effector genes into a target population [82]. Thus, CRISPR-based gene drive technology seems to have arrived at a difficult crossroads: how to safely field-test a system that by its very nature requires so few individuals to initiate the irreversible invasion of a target population?

Engineering requirements for Safe drive: The advantage of targeting maleness

Ten years ago, James [83] laid out a set of engineering challenges that would have to be overcome to realize the potential for using a gene drive approach to achieve fixation of a synthetic allele in a wild target population. These included challenges of genetic engineering (drive mechanism must be sufficient to overcome fitness costs, must work rapidly, be adaptable to many species, be re-useable, and retain linkage to any cargo), epidemiology

(gene drive system must not change the behavior of the target organism in such a way as to contribute to the emergence of new pathogens or disease), and ecology (must not adversely affect non-target organisms) as well as social and political considerations. Site-specific nuclease-based gene drive systems were shown to be sufficiently powerful and rapid at driving a gene into an unstructured cage population [67]; the substitution of CRISPR/Cas9 for HEGs ensures that such drives are both highly adaptable and highly reusable.

Partnerships with supportive governments, local collaborators and a willing public are crucial to establishing field-based testing in environmentally relevant areas (essentially, where releases might occur) [84]. Modeling suggests that a powerful nuclease-based gene drive system could become established in the wild with the accidental release of just a few individuals during such testing [85], though the outcome may be highly dependent on local landscapes and vector population density and dispersal characteristics [86]. This means that securing permission for field-based testing of highly-active (CRISPR) gene drive constructs may be challenging in epidemiologically relevant (dengue or malaria-endemic) areas without guarantees that the introduced gene could be removed from the study area if needed. The successful drive of CRISPR/Cas9 into a gene critical only to females is predicted to result in decrease in the overall population due to lack of females [38]; the same result might be obtained by driving a maleness gene such as *Nix* into a population [34]. In a completely homogenous population, such as a laboratory or field cage trial, this could be expected to lead to complete extinction of the target population. However, modeling that takes into account spatial variability in population structure and stochasticity suggests that a highly invasive gene such as a HEG or CRISPR/Cas9 may drive itself to extinction locally prior to extinction of a discontinuous target population, in a manner that is highly dependent on the dispersal characteristics and abundance of the target population, as well as the release strategy (small number of large releases vs. large number of small releases) [86]. A similar stochastic loss of introduced transgenes is predicted for female-specific RIDL (Release of Insects with a Dominant Lethal) [87] as well as so -called ‘reduce and replace’ [88] strategies where the vector population is first suppressed, followed by mass releases of an alternative genotype (carrying an engineered or naturally occurring resistance gene). Thus, gene drive strategies based on population suppression may be tuned to be self-limiting, though more detailed modeling is needed in this area in regards to sex manipulation. Though expected to persist in the environment longer than purely sterile insects, the lower predicted operational costs (based on reduced mass rearing capacity and/or sex separation) of implementing and maintaining a vector control program incorporating driving ‘maleness’ into a vector population is an attractive option worth continued pursuit and may not present the same ethical challenges associated with the permanent modification or elimination of wild populations. As in most gene drive systems, mutations in the effector (the ‘maleness’ gene) may result in the spread of a non-functional allele. However, this concern is not as severe as in conventional gene drive systems because the maleness gene is not expected to be sustained in the population in perpetuity and thus has less time to accumulate mutations. Nonetheless, such a concern may be mitigated by incorporating multiple copies of the maleness gene or genes with redundant functions.

Concluding remarks

Mosquitoes are highly sexually dimorphic. For the major vectors of malaria and dengue there is also a clear separation between harmless, nectar-feeding males and deadly bloodfeeding females. The identification of molecular switches and genetic programs that control the decision made in the early developing mosquito embryo to proceed as male or female may be used to improve existing sterile insect strategies for controlling mosquito-borne disease agents. Combined with the sudden prospects for easily programmable CRISPR-based gene drive systems, master regulators of sex may be developed as self-propagating systems for driving maleness into wild populations. Such strategies may be more efficient than sterile insect releases, but may still ultimately be self-limiting as local population crash due to insufficient females could eliminate the engineered transgene from the environment. Though some details are emerging, more work is needed to understand the molecular mechanisms of sex and how manipulations of this phenotype could be used in vector control.

Acknowledgements

This work was supported by the NIAID (AI113643, AI105575, and AI077680) and its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIAID. We thank Brantley Hall for drafting Figure 1.

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Box 1. Nuclease-based gene drive systems depend on host DNA repair factors

While programmable nucleases themselves appear to get all the attention, their contribution to gene drive is merely to introduce a DSB at a predetermined location in a genome. To actually achieve gene drive requires that this break be repaired using the sister chromatid or homologous chromosome as a template, a process termed homology-dependent repair (HDR) (reviewed in [89]). However, other cellular repair pathways such as non-homologous end-joining (NHEJ) and single-strand annealing (SSA) compete for access to the DSB [90]. NHEJ is initiated when the Ku complex binds to the free DNA ends and recruits a complex containing Ligase4; subsequent ligation can result in small insertions or deletions at the break site. A competing complex, termed MRN, initiates resection of one strand at both ends of the break [91], subsequently recruiting various additional single-stranded exonucleases to the break site (reviewed in [92]). At this point, other factors are recruited to the break site and use the now single-stranded tails to perform a homology search. If an acceptable template is found on the homologous chromosome, then HDR results in gene conversion (gene drive).

Trends Box

- Predominantly male progeny may be obtained when mosquito sex-determination is manipulated, either by knocking down the female-specific transcript of *dsx* in *Aedes aegypt*, or by expressing an endonuclease that specifically destroys the X chromosome during spermatogenesis in *Anopheles gambiae*.
- The first insect M factor, *Nix*, has been discovered in *Ae. aegypti*. Ectopic expression of *Nix* is sufficient to initiate male development in genetic females. Two candidate M factors have been discovered in Anopheles mosquitoes. M factors and other intermediary genes in the sex-determination pathway provide new ways to promote “maleness”, the ultimate disease-refractory trait in mosquitoes.
- CRISPR/Cas9-based gene drive system has been successfully demonstrated in *Drosophila melanogaster*, which could be developed in mosquitoes to self-propagate dominant-acting maleness genes.

Outstanding Questions Box

- Questions related to sex-determination pathway in *Aedes aegypti:* Is NIX a splicing factor? What are the RNA targets of NIX? Does NIX directly regulate the splicing of the *dsx/fru* pre-mRNA, or does NIX function through other intermediates? Is there a *tra*-like gene in *Ae. aegypti* and if so is its processing regulated by NIX?
- Questions related to sex-determination pathway in *Anopheles* mosquitoes: Is *Guy1* or *gYG2* the M factor in *An. stephensi* and *An. gambiae*, respectively? Are sex-determination and dosage compensation regulated by the same primary signals in Anopheles mosquitoes?
- Questions related to *Nix* evolution: How widely is *Nix* distributed? Are there other genes in addition to (or instead of) *Nix* in the M-loci of the other Culicinae species? Can *Nix* found in other species functions as an M factor?
- Will ectopic expression of a *Nix* transgene convert genetic females into fertile males, sterile males, or simply dead females? Can mosquito M factors and other intermediary genes in the sex-determination pathway facilitate genetic methods to control mosquito-borne infectious diseases by improving sex-separation or introducing male-bias?
- Can CRISPR/Cas9-based gene drive system be developed in mosquitoes? Can we use such an easily-programmable gene drive to drive “maleness” into mosquito populations? Will such a strategy indeed be self-limiting and thus more feasible from a regulatory perspective and yet more efficient than sterile insect releases?

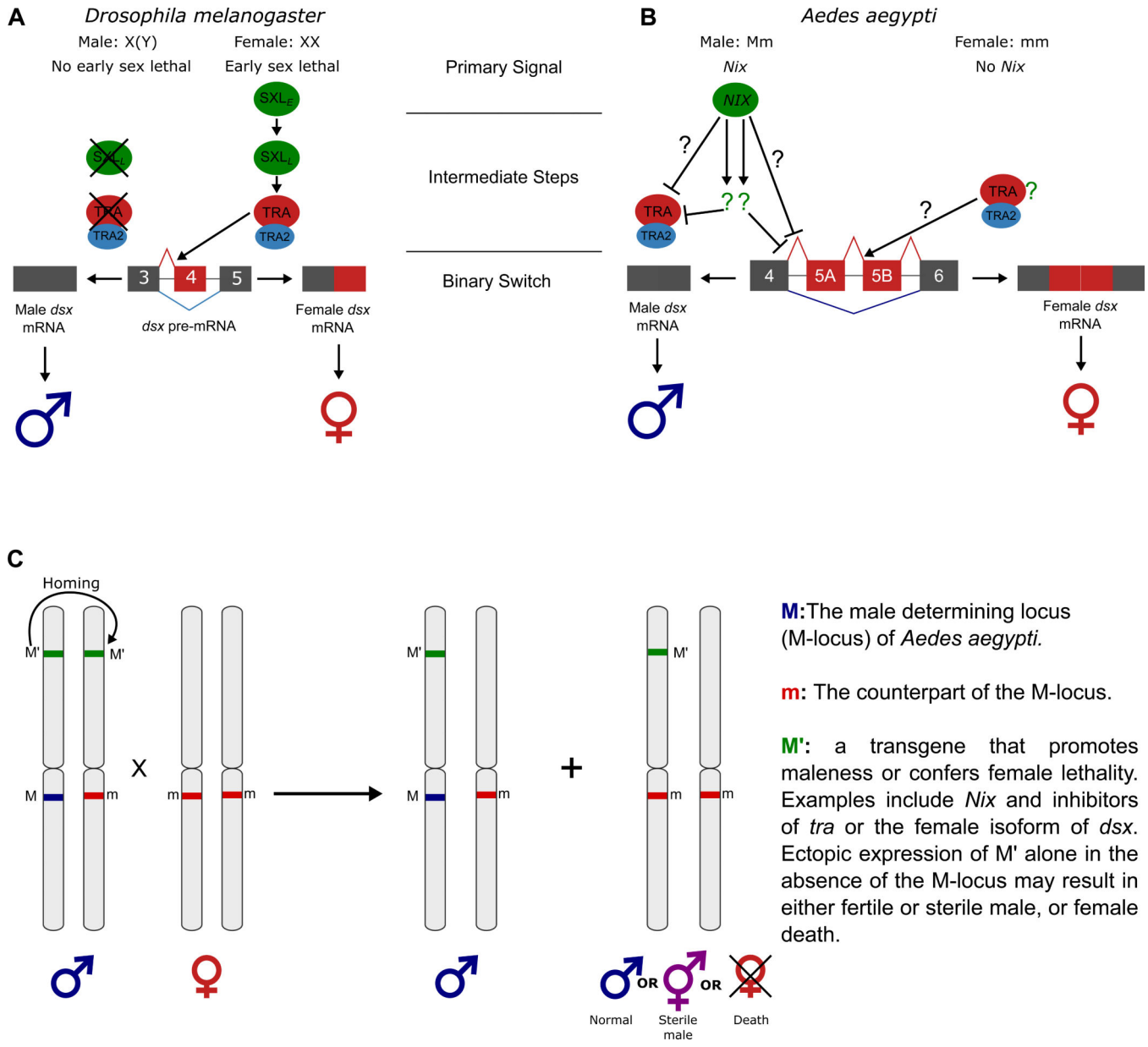

Figure 1. Sex determination pathways

(A) *Drosophila melanogaster*. (B) *Aedes aegypti*. (C) Consequences of homing of a maleness gene in *Ae. aegypti*. The cascade of events leading to sex-specific splicing of *doublesex* (*dsx*), one of the two key transcription factors that program sexual differentiation, is shown. Sex-specific splicing of *fruitless* (*fru*), the other key transcription factor, is not shown. Ovals indicate protein products and green question marks represent possible unknown gene products. Black questions marks indicate possible interactions. Boxes represent *dsx* exons and the numbers indicate their order. The inhibition and activation arrows pointing to exon-intron junctions indicate the inhibition of a strong or promotion of weak splice site, respectively. SXL_E, early sex-lethal protein that is expressed from the early embryonic *sxl* promoter; SXL_L, late sex-lethal protein; TRA and TRA2 form a complex that regulate the splicing of *dsx* and *fru*. Transformer (TRA) confers sex-specificity when in

complex with Transformer 2 (TRA/TRA2). Homing refers to the general process where a gene is duplicated onto its homologous chromosome, for instance driven by a homing endonuclease or CRISPR/Cas9.

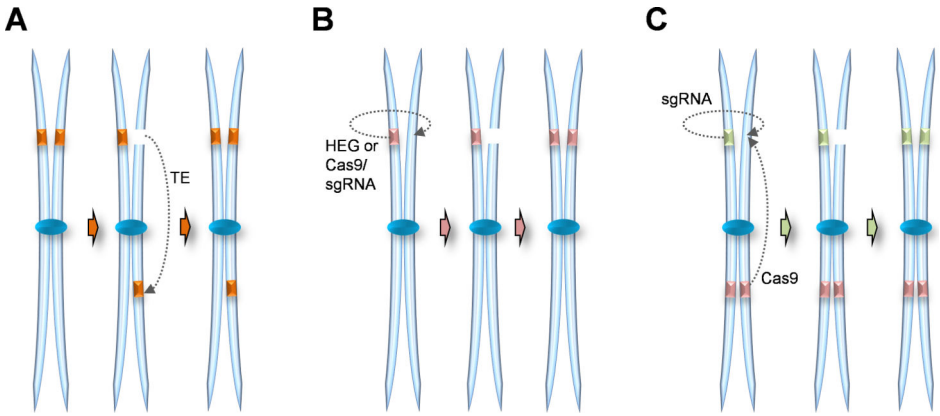

Figure 2. Gene drive systems based on homology-dependent repair

(A) Transposable element (TE) mobilization leaves behind an open double-stranded DNA break. (B) Site-specific integration of a homing endonuclease (HEG) or Cas9/sgRNA into its own target site can be followed by cutting of the homologous chromosome. (C) Fixation of germline-expressed active Cas9 can result in the drive of any subsequent guide RNA if placed at its target site. In all cases, if DNA break induction occurs while the sister chromatid or homologous chromosome is present (mitotic divisions of germline stem cells or during meiosis), homology-dependent repair can regenerate the TE, HEG or Cas9 system resulting in a net increase in copy number.