Targeting the X Chromosome during Spermatogenesis Induces Y Chromosome Transmission Ratio Distortion and Early Dominant Embryo Lethality in Anopheles gambiae

Nikolai Windbichler*, Philippos Aris Papathanos*, Andrea Crisanti*
Faculty of Life Science, Imperial College London, London, United Kingdom

Abstract

We have exploited the high selectivity of the homing endonuclease I-PpoI for the X-linked Anopheles gambiae 28S ribosomal genes to selectively target X chromosome carrying spermatozoa. Our data demonstrated that in heterozygous males, the expression of I-PpoI in the testes induced a strong bias toward Y chromosome–carrying spermatozoa. Notably, these male mosquitoes also induced complete early dominant embryo lethality in crosses with wild-type females. Morphological and molecular data indicated that all spermatozoa, irrespectively of the inheritance of the transgene, carried a substantial amount of I-PpoI protein that could attack the maternally inherited chromosome X of the embryo. Besides the obvious implications for implementing vector control measures, our data demonstrated the feasibility of generating synthetic sex distorters and revealed the intriguing possibility of manipulating maternally inherited genes using wild-type sperm cells carrying engineered endonucleases.

Introduction

Mosquitoes represent a major and global cause of human suffering due to the infectious agents they transmit. About two million people die from mosquito-borne diseases every year. These include parasitic infection, i.e. malaria and filariasis and viral diseases such as dengue, encephalitis and yellow fever. Malaria alone, transmitted exclusively by Anopheles mosquitoes carrying Plasmodium protozoan parasites, causes the death of more than a million people each year, most of which are occurring in sub-Saharan Africa [1]. Rather than being under control, the threat represented by mosquitoes is increasing due to the inadequacy of existing control measures in the developing world and the progressive spread of insecticide-resistant insects [2].

Gene manipulation technologies promise to dramatically enhance the development of novel control measures against vector-borne diseases [3]. Different approaches are being investigated including the development of disease-refractory mosquitoes to implement population replacement strategies [4–8]. Genetic sterility, genetic drive systems or the release of insects carrying a dominant lethal gene (RIDL) have been suggested as possible strategies to reduce population density [9–14]. A number of reports have shown how genetically manipulated mosquitoes can provide valuable solutions to overcome ineffective, costly and time-consuming steps that have previously hampered vector control measures involving the sterile insect technique [15] (SIT). These include the use of genetic markers to monitor both male dispersal and mating competitiveness and the separation of male and female mosquito larvae at an early developmental stage to address the requirement to release only male mosquitoes, as females contribute to disease transmission [16]. An inducible genetic sterility system, designed to overcome the fitness cost associated with chemical and irradiation sterilization, has been developed in Drosophila as a proof of principle [17].

A novel mechanism has recently been proposed to distort the sex ratio in natural populations based on the use of engineered mosquitoes expressing a homing endonuclease enzyme targeting X chromosome carrying spermatozoa, thereby generating an excess of spermatozoa carrying chromosome Y [13,18]. It has long been recognized that if the Y chromosome were to show transmission ratio distortion and spread in a population, then the sex ratio would become male biased and the population could ultimately go extinct [19,20]. Natural driving Y chromosomes in Aedes aegypti and Culex pipiens have been described and can produce extreme sex ratios of more than 90% males in each generation [21]. Although the molecular details of how these distorters act are unknown, cytological evidence suggests that they are associated with breaks in the X chromosome during male meiosis I [22,23]. A similar system for sex ratio distortion could be artificially created using the I-PpoI homing endonuclease: this enzyme has been shown to selectively cleave the ribosomal rDNA repeats in the A. gambiae Sua-4.0 cell line, leading to nucleolar fragmentation and cell death.
Author Summary

*A. gambiae* mosquitoes are the main vectors of human malaria. The inadequacy of existing control measures for these mosquitoes has prompted research into methods for genetic control. We have genetically engineered *A. gambiae* mosquitoes to express, during spermatogenesis, an enzyme that selectively cuts a DNA sequence present only on a family of essential genes located on the X chromosome. We found that in heterozygous male mosquitoes, this genetic modification induced complete early dominant embryo lethality in crosses with wild-type females. All spermatozoa from these males, including those not containing the genetic modification, carried the chromosome X cutting enzyme that could attack the maternally inherited X chromosome of the embryo. Furthermore, this genetic modification introduced a strong, negative bias toward X chromosome–carrying spermatozoa. These transgenic mosquitoes fulfilled a number of requirements for implementing vector control measures based on genetic sterility, but our data also demonstrate the feasibility of generating synthetic sex distorters and reveal the possibility of manipulating maternally inherited genes using wild-type sperm cells carrying enzymes designed to attack selected maternal DNA sequences.

[18]. In several anopheline species, including at least two members of the *A. gambiae* complex, the rDNA repeats are exclusively located in the centromeric region of chromosome X [24–26]. Accordingly, the expression of I-PpoI during spermatogenesis is anticipated to incapacitate X chromosome carrying spermatozoa and induce sex ratio distortion. This mechanism would provide a formidable tool to distort the ratio in favour of males, thereby leading to the reduction or eradication of field populations.

We have engineered male *A. gambiae* mosquitoes to express, during spermatogenesis, the I-PpoI homing endonuclease as a fusion protein with the eGFP fluorescent marker with the aim of inducing sex ratio distortion, combined with the expression of an early developmental marker for sexing. We report here on the induction of sex ratio distortion, combined with the expression of an fusion protein with the eGFP fluorescent marker with the aim of targeting marker and revealing the possibility of manipulating maternally inherited genes using wild-type sperm cells carrying enzymes designed to attack selected maternal DNA sequences.

Results

Identification of I-PpoI Recognition Sequences in the *A. gambiae* Genome

The I-PpoI recognition sequence consists of a 15bp core motif flanked by a number of additional nucleotides that also contribute to the overall efficiency of the endonuclease binding and cleavage activity [27]. We searched the *A. gambiae* genome for the presence of the cognate I-PpoI recognition site. This analysis revealed a match to the wild type 15 base pair core recognition sequence outside the rDNA genes which were recently mapped to contig AAAB01008976 of the chromosome X of *A. gambiae* [28]. We also screened the genome for sequences matching a number of previously described recognition sequence variants that were identified by in vitro selection to be efficiently cut by I-PpoI [29]. None of these variants were found in the *A. gambiae* genome, even when taking into account only the 15bp core recognition sequence. Recently a complete specificity profile of I-PpoI has been established [N. Nomura, personal communication]. Utilizing this profile we screened the *A. gambiae* genome for any recognition site variants predicted to be cut by I-PpoI with high efficiency.

Again this analysis did not reveal matches in the *A. gambiae* genome assembly. We therefore concluded that the X-linked multi-copy rDNA locus, containing the complete I-Ppo1 core and flanking recognition sequence, would be the main predicted target locus of I-Ppo1 in the *A. gambiae* genome.

Generation of Transgenic Mosquitoes Expressing I-Ppo during Spermatogenesis

We injected mosquito embryos with the transformation construct pBac[3xP3-DsRed]β2-eGFP::I-Ppo1 that was designed to direct the expression of I-Ppo1 in the testis during the process of spermatogenesis (Figure 1A). The structural properties of I-Ppo1 and eGFP allow the generation of a fusion protein that maintains both the activity and the selectivity of the endonuclease [18], while functioning as a visible marker for mosquito sexing during larval development. We utilized a shortened version of the β2 tubulin 5’ and 3’ regulatory regions [30] to direct expression of eGFP::I-Ppo1. Previous studies have demonstrated that the β2 promoter is exclusively activated in male gonads and it can be utilised to selectively direct the expression of eGFP to the male gonads of anopheline mosquitoes [16]. The construct also contains piggyBac inverted repeats and the DsRed gene under the control of the 3xP3 promoter as a germine transformation marker (Figure 1A). Two independent transgenic lines (β2Ppo1 and β2Ppo2) were obtained in independent sets of embryo injections. Molecular analysis showed that each line resulted from a single integration event. Inverse PCR, followed by sequencing of the regions flanking the integration event, revealed that the construct had integrated at position 49029419 on chromosome 2L and position 11872203 on chromosome 3R in the lines β2Ppo1 and β2Ppo2, respectively (Figure S1). Both lines showed a strong green fluorescent signal localised in the male gonads, visible from late third instar larvae throughout adult development, thus indicating that the eGFP::I-Ppo1 fusion protein was exclusively expressed in the testes of male larvae, pupae and adults (Figure 1B).

To confirm this conclusion we searched for the presence of *in vivo* I-Ppo1 activity in the testes of β2Ppo mosquitoes. We analysed, by southern blot hybridization experiments, the integrity of the 28S rDNA genes in DNA extracted from β2Ppo mosquito testes (Figure 1C). Our results showed that, when using DNA from wild type mosquitoes, the probe containing the 28S rDNA sequence hybridized to a band of about 2.9 kb, in agreement with the size of the ribosomal gene and the position of the Cld sites. Larger fragments were also recognized due to heterogeneity in the 28S ribosomal genes [19]. The same experiment carried out using DNA extracted from the testes of β2Ppo1 males showed the presence of a smaller band of 1.4kb, in agreement with the position of the I-Ppo1 recognition site in the ribosomal genes (Figure 1C and Figure S1). The same digestion product was observed when DNA extracted from wild type [WT] testes was treated with recombinant I-Ppo1 in *in vitro* conditions (Figure 1C). In addition, *in vitro* conditions recombinant I-Ppo1 digested a 2kb PCR product encompassing the 28S rDNA probe fragment into a 1kb product. These results indicated that in the testes of transgenic males, the eGFP::I-Ppo1 fusion protein was able to cleave the 28S rDNA on chromosome X.

β2Ppo Testes Develop Normally and Generate Spermatozoa Containing Active eGFP::I-Ppo1

We analyzed the development of the testes and spermatogenesis in heterozygous β2Ppo males using fluorescent microscopy and 3D imaging. Our results indicated that the testes were morphologically indistinguishable from those of wild type mosquitoes. The testes of β2Ppo1 mosquitoes showed a typical eGFP fluorescence...
pattern that reflected the direction of the differentiation process and the activational timing of the β2 tubulin promoter from sperm germ cells to mature spermatozoa. Confocal analysis of β2Ppo1 testes showed both mature sperm cells being produced in spermatocysts and spermatozoa reaching the male vas efferens (data not shown). In mating experiments with virgin wild type females, β2Ppo spermatozoa were found to be successfully transferred into the spermathecae (Figure 2A). All these spermatozoa showed a variable degree of green fluorescence localized to their nuclei, thus indicating they were carrying along substantial amounts of eGFP::I-PpoI fusion protein (Figure 2A). To investigate this phenotype in more detail we analyzed, by confocal microscopy and 3D-image reconstruction, the DNA content, the nuclear volume and the intensity of eGFP fluorescence of β2Ppo spermatozoa. Spermathecae from wild type females mated to either heterozygous β2Ppo2 or wild type males were dissected, fixed and stained with DAPI. Sperm were released and nuclei of about one hundred spermatozoa were individually analyzed for each cross. Wild type spermatozoa showed undetectable amounts of green fluorescence and a homogenous DAPI signal, whereas all spermatozoa from heterozygous β2Ppo2 males showed eGFP fluorescence signal which was mainly localized to the nuclei (Figure 2B). This analysis also indicated a moderate variability in the DAPI staining of β2Ppo spermatozoa (Figure 2A). The distribution of the eGFP fluorescence in β2Ppo spermatozoa is in agreement with both the transcription pattern of the β2 tubulin promoter in the testes and the structure of the spermatocyte: transcription from the β2 tubulin promoter starts shortly before the first meiotic division and continues throughout the subsequent stages of spermatozoa differentiation. Furthermore, in both insects and mammals, all spermatozoa derived from a single spermato-gonial cell are connected through cytoplasmic bridges [31] to form a spermatocyst for a period of time that largely coincides with the temporal activity of the β2 tubulin promoter. This allows the sharing of cytoplasmic constituents between developing spermatozoa and would provide I-PpoI protein a means to migrate from cell to cell. Therefore, all spermatozoa, irrespective of whether they will inherit the transgene or not, are anticipated to carry along the eGFP::I-PpoI fusion protein.

β2Ppo Heterozygous Males Induce Early Embryo Lethality

To investigate whether the expression of I-PpoI had an effect on fertility and/or sex ratio distortion of the progeny, we crossed heterozygote β2Ppo1 males with WT females. As a control, heterozygote β2Ppo1 females were crossed with WT males.

Figure 1. Transformation construct and expression of I-PpoI in the testes of transgenic mosquitoes. (A) Schematic representation of the construct pBac[3×P3-DsRed]β2-eGFP-I-PpoI containing the left and right piggyBac inverted repeats (pBacR,L); the Pax promoter (3×P3) driving the DsRed marker gene; and the eGFP::I-PpoI effector gene (eGFP I-PpoI) under the control of β2 tubulin promoter and terminator (β2). (B) Transmission and fluorescent images of dissected adult testes, larval head and pupa of β2Ppo1 male mosquitoes. (C) Southern blot analysis of the 28S ribosomal DNA locus. DNA from testes of WT (lanes 1 and 3) and β2Ppo1 males (lanes 2 and 4) was digested with the endonuclease ClaI in vitro and hybridized with a probe encompassing the 28S ribosomal gene (Figure S1). As control both the DNA extracted from the WT and β2Ppo1 testes was treated with recombinant I-PpoI as indicated. Furthermore the PCR product (2kb) used as probe either treated with recombinant I-PpoI or untreated was analysed under the same hybridization conditions (lanes 4 and 5). Open and filled arrowheads indicate the full length and digested rDNA fragments respectively.

doi:10.1371/journal.pgen.1000291.g001
Females were allowed to lay eggs on two consecutive occasions after they were blood fed. These experiments indicated that female $\beta_2Ppo1$ mosquitoes did not show any anomalies in terms of fertility when crossed to WT males. These mosquitoes, compared to females of WT crosses, laid a normal number of eggs with comparable hatching rate, pupal development and adult sex ratio (Table 1 and data not shown). In contrast, while WT females crossed to $\beta_2Ppo1$ males produced normal numbers of eggs, these eggs failed to hatch. These experiments were performed with $\beta_2Ppo1$ mosquitoes of different generations (Table 1), as well as with line $\beta_2Ppo2$ which showed identical properties (Table S1). Both transgenic lines, $\beta_2Ppo1$ and $\beta_2Ppo2$, have now been backcrossed to WT males for 14 and 16 generations respectively, and the males originating from these crosses were tested for fertility in each generation. Throughout this period no phenotype other than total male sterility was observed (data not shown).

To analyze the nature of male sterility in the $\beta_2Ppo1$ and $\beta_2Ppo2$ lines, we investigated whether the spermatozoa from these mosquitoes had fertilized the eggs in crosses with WT females and to establish the timing of embryo developmental arrest. For this purpose the embryos were fixed 24 hrs post oviposition, the chorion removed and the DNA stained with DAPI to highlight the localization and distribution of cell nuclei using confocal microscopy. In most of the embryos examined at 24 hrs after oviposition we could only identify two DAPI stained bodies. The first body was localized in the central anterior region of the embryo, while the second body was found in the anteroventral region in close proximity to the micropyle, thus suggesting their identification as the female and male pronuclei respectively (Figure 3A,B). A few embryos showed features of cellularization and nuclear division that did not progress to larvae formation. We also used confocal analysis to compare the size of sperm nuclei and
the male pronuclei originating from β2Ppo males. This analysis showed that while the diameter of the sperm nuclei ranged from 2 to 4 μm, that of the male pronuclei was bigger (7–8 μm), suggesting that the latter had undergone decondensation. Male pronuclei were also stained with an antibody directed against H2AX, associated with DNA double strand breaks [32]. This confirmed the transport of the fusion protein into the embryo and revealed the presence of DNA double strand breaks in the maternal genome (Figure 3B). Control experiments on WT embryos did not show nuclear reactivity with either the anti eGFP or the anti γ-H2AX antibody (12 of 28 nuclei examined) that reacts with phosphorylated histone H2AX, suggesting that the latter had undergone decondensation. Male pronuclei were also stained with an antibody directed against eGFP (28 of 28 nuclei examined) while female pronuclei were found to stain with an anti eGFP (28 of 28 nuclei examined) that reacts with the β2 tubulin promoter. Our results also demonstrated that in the β2Ppo male mosquitoes. In other experiments we used a primeset designed to amplify a sequence that in previous reports was shown to specifically detect the A. gambiae Y chromosome [33]. Notably, in 88% of the embryos examined we amplified the diagnostic band for the Y chromosome in vivo. Intriguingly, heterozygous β2Ppo males were completely sterile. To understand the molecular basis of this phenotype, we investigated whether the expression of I-PpoI disrupted the process of spermatogenesis or impaired the ability of spermatozoa to enter eggs. Microscopy analysis indicated that the testes of β2Ppo males had spermatocytes morphologically

Mating Competitiveness of β2Ppo Males in Laboratory Cage Experiments

With the aim of assessing the suitability of the β2Ppo1 and β2Ppo2 transgenic lines for SIT, we analyzed whether β2Ppo2 males could successfully compete with WT mosquitoes for mating partners in laboratory cage experiments. In these experiments identical numbers of WT and β2Ppo2 males were allowed to mate with varying numbers of WT virgin female mosquitoes. Five days later we collected the females and analyzed the spermatheca for the presence of either WT or β2Ppo spermatozoa in multiplex PCR experiments. We utilized a PCR primer pair amplifying the Y specific sequence to assess the mating rate of the females and a second primer pair amplifying the sequence of the I-PpoI transgene, to determine the number of females mated with β2Ppo2 males. Our data showed that the I-PpoI sequence could be amplified in a substantial proportion of female spermathecas, ranging from 48 to 56%, at different female to male ratios, thus suggesting that the transgenic males were not impaired in their ability to mate with WT females (Figure 2C).

| Table 1. Outcome of crosses between transgenic β2Ppo and WT mosquitoes. |

| G2 crosses | Eggs laid | Larvae hatched | Screened | Transgenic | % Transgenic |
|------------|-----------|----------------|----------|------------|-------------|
| 10♂ x 30♀ β2Ppo1 | Lay 1 | 658 | 0 | - | - |
| 20♂ x 17♀ | Lay 1 | 610 | 544 | 152 | 74 | 48.6% |
| 25♂ x 25♀ | Lay 1 | 121 | 713 | 0 | - | - |
| 25♂ x 25♀ | Lay 1 | 1024 | 878 | 798 | 414 | 51.8% |

Heterozygote β2Ppo1 males of generations 2 and 3 were crossed to WT females. As control β2Ppo1 heterozygote females of generation 2 and 3 were crossed to WT males. The total number of eggs laid and larvae hatched are shown for two consecutive egg depositions (Lay1 and Lay2). In addition larvae originating from control crosses were screened for the 3xP3-DsRed marker to determine the numbers of WT and transgenic offspring as indicated. doi:10.1371/journal.pgen.1000291.t001

With the aim of assessing the suitability of the β2Ppo1 and β2Ppo2 transgenic lines for SIT, we analyzed whether β2Ppo2 males could successfully compete with WT mosquitoes for mating partners in laboratory cage experiments. In these experiments identical numbers of WT and β2Ppo2 males were allowed to mate with varying numbers of WT virgin female mosquitoes. Five days later we collected the females and analyzed the spermathecae for the presence of either WT or β2Ppo spermatozoa in multiplex PCR experiments. We utilized a PCR primer pair amplifying the Y specific sequence to assess the mating rate of the females and a second primer pair amplifying the sequence of the I-PpoI transgene, to determine the number of females mated with β2Ppo2 males. Our data showed that the I-PpoI sequence could be amplified in a substantial proportion of female spermathecas, ranging from 48 to 56%, at different female to male ratios, thus suggesting that the transgenic males were not impaired in their ability to mate with WT females (Figure 2C).

Discussion

We have generated two independent A. gambiae lines, β2Ppo1 and β2Ppo2, carrying the construct pBac{3xP3-DsRed}β2-eGFP-I-PpoI in distinct regions of the genome. Males originating from crosses between heterozygous β2Ppo females and WT males showed, starting from late 3rd instar larvae, a strong green fluorescence signal exclusively localized in the testes, indicating that the I-PpoI fusion protein was being produced in spermatocytes according to the anticipated expression pattern of the β2 tubulin promoter. Our results also demonstrated that in the testes of β2Ppo males ribosomal DNA was cleaved at the I-PpoI site. This finding indicated that the endonuclease component of the eGFP-I-PpoI fusion protein retained its ability to cut the X chromosome in vivo. Intriguingly, heterozygous β2Ppo males were completely sterile. To understand the molecular basis of this phenotype, we investigated whether the expression of I-PpoI disrupted the process of spermatogenesis or impaired the ability of spermatozoa to enter eggs. Microscopy analysis indicated that the testes of β2Ppo males produced spermatozoa morphologically
identical to those of WT mosquitoes. Dissection of female mosquitoes mated with b2Ppo males indicated that the spermatozoa had been successfully transferred to the spermathecae. Furthermore, confocal microscopy showed the presence of both the female and male pronuclei in the embryos. Taken together, these experiments demonstrate that b2Ppo males produced competent and viable spermatozoa. Nuclear fluorescence staining also revealed that the embryos originating from b2Ppo males were arrested very early in their development, probably at a point before the fusion of the male and the female pronuclei.

The genetic study of these embryos provided clues to formulate a molecular explanation for the early dominant lethality induced
by β2Ppo males. Although spermatozoa carrying the transgene had fertilized only half of the eggs, as inferred by PCR genotyping analysis, confocal microscopy and 3D imaging demonstrated that all spermatozoa showed some level of eGFP fluorescence. This observation is in agreement with the temporal expression of the β2tubulin promoter and the structure of the spermatogonial syncytium. Importantly, it also indicates that all spermatozoa of β2Ppo males could deliver active nuclear I-PpoI protein into the embryos, thereby inducing DNA damage to the maternal inherited chromosome X. This provides an explanation for the dominance of the lethality phenotype. This notion was also supported by findings showing that individual male nuclei within the developmentally arrested embryos stained positive with anti-eGFP antibodies, while the female pronuclei did not. In contrast antibodies directed against γ-H2AX indicated double strand DNA damage only on the female pronuclei.

The genetic analysis of the embryos also revealed that more than 80% had originated from spermatozoa carrying the Y chromosome, thus indicating that although the expression of eGFP::I-PpoI did not impair the process of spermogenesis or the viability of sperm cells in general, it did selectively target X chromosome carrying spermatozoa, thereby causing transmission ratio distortion. It is possible that the remaining embryos had been fertilised by spermatozoa lacking both chromosomes X and Y. Although of no immediate practical application due to embryo lethality, these results demonstrate that synthetic sex distortion mechanisms can be developed. Both mathematical modelling and the study of naturally occurring sex distorters in some insect species predict that, if linked to the Y chromosome, such distorters would represent extremely powerful tools to knock down a target population in a relatively short time.

The development of the transgenic lines β2Ppo1 and β2Ppo2 has some direct implications for the implementation of vector control measures based on genetically modified mosquitoes. Both lines meet a number of desirable requirements for SIT; including: i) a visible marker for monitoring male dispersal and competitiveness, ii) a validated sexing system that can be effectively automated; and iii) complete and dominant genetic male sterility. Laboratory cage experiments performed here indicate that β2Ppo male mosquitoes are not impaired in their ability to mate with WT females when mixed with WT males. Since the I-Ppo1 recognition site is located in the 28S rDNA gene in a highly conserved rRNA region, which forms the peptidyl transferase centre of the ribosome, the approach described here could be applied to other pest species.

The finding that non-genetically modified spermatozoa can carry along effector molecules selectively targeting the maternal genome offers the possibility to develop “Medea”- like cytoplasmic incompatibility systems predicted to have strong driving properties. Finally, our results reveal the intriguing possibility of manipulating maternally inherited mosquito genes involved in parasite transmission or sex determination by using wild type sperm cells carrying engineered endonucleases such as HEGs or zinc fingers. To this end, heterozygous transgenic males can be produced that express, during the process of spermogenesis, a rare-cutting endonuclease engineered to selectively target such genes. Our findings demonstrate that endonuclease protein will be transferred to all spermatozoa irrespectively of the segregation of the transgene itself and therefore will be transported into the wild type embryo at the time of fertilization. The endonuclease will introduce changes into the targeted maternal sequence by cleavage followed by non-homologous repair. A fraction of the embryos will inherit these endonuclease-induced changes without carrying the original transformation construct. The resulting mosquitoes would address a number of safety and environmental issues associated with the release of genetically manipulated mosquitoes for vector control as they will not contain a selectable marker or a transformation construct.

### Materials and Methods

#### Plasmid Construction

The 1.2kb eGFP::I-PpoI cassette was amplified from pEGFP-nPpo [18] using primers PpoI34h2f and GCCGAAGCAAGGGCGGAGGCTGTTC and PoopI42h2r GTCCGACTTATACGACAAAGTGACTGCCCCTTTGTGTG. A 1.7kb β2ubulin GFP cassette was amplified from pPB[ΔDsRed]β2-EGFP [16] using primers b2sAscIfw AAGGGCCGCCCTAGGTGCATTATACTGTA and b2sAscirev AAGGGCCGCCGATTTAAGGACCGATTGC and cloned into the shuttle vector pSlfa180fa [34] using AscI. From this vector the original GFP was removed with HindIII and replaced by the eGFP::I-PpoI cassette cut with HindIII. The resulting 2.3kb cassette contains the nuclear localization signal between the N-terminal eGFP and the C-terminal I-Ppo1 coding regions which are flanked by the β2 regulatory regions and was moved into the pPB[ΔDsRed] backbone using AscI to create pBac{3xP3-DsRed}β2-eGFP::I-PpoI.

#### Development of Transgenic Lines

Transgenic lines were developed as described [16,35]. *A. gambiae* (strain G3) embryos were injected using a Femtojet Express injector and sterile Femtotips (Eppendorf) with a mixture of 0.2 μg/μl of plasmid and 0.8 μg/μl of piggyback helper RNA. The hatched larval survivors were screened for transient expression of the 3xP3 DsRed marker and only transients were grown up and crossed to wild-type mosquitoes. The progeny of these crosses was analyzed for DsRed fluorescence. To establish line β2Ppo1 we injected 430 embryos from which 42 (9.7%) survivors hatched 21 (50%) of which showed transient expression of the marker. 6 female transients survived to adulthood and when crossed to WT gave rise to 1 transgenic female individual. To establish β2Ppo2 we injected 241 embryos and obtained 45 (18.6%) survivors including 30 transients (66.6%). 11 female transients survived to adulthood and when crossed to WT gave rise to 8 transgenic individuals (2 males, 6 females) from one founder. Females were crossed separately to WT males and molecular analysis of their progeny confirmed that they had originated from a single integration event. Transgenic mosquitoes at different developmental stages were analyzed on a Nikon inverted microscope (Eclipse TE200) to detect eGFP and DsRed expression. Digital images were captured on a Nikon inverted microscope (Eclipse TE200) with an attached Nikon DXM1200 digital camera. The β2Ppo lines were reared in a way so that in each generation transgenic mosquitoes were separated into males and females and crossed back to WT *A. gambiae* G3.

#### Southern Blot

Genomic DNA was digested with CiaI in the presence and absence of I-PpoI. As a probe we used a 2 kb rDNA PCR fragment amplified from genomic DNA using the primers rDwd GCCGAAGCAATTAGCCTTTAAAATGAGATG and rDrev CACCACTAGGTTAAAATAACCCAGTGTCTCACG. The probe was labelled with P32 using the High Prime DNA labelling kit (Roche) and purified with ProbeQuantTM G-50 columns (GE Healthcare). Results were visualized using a FUJIFLMFLA-5000 Phosphoimager (Fuji Photo Film Co. Ltd, Stamford, CT, USA). For in vitro digestions, we used commercially available I-PpoI (Promega) enzyme.
Analysis of Sperm Nuclei Recovered from Spermatheca

Virgin females mated with WT males or transgenic males were dissected in PBS. Spermathecae were checked for the presence of sperm on a widefield microscope. Spermathecae containing sperm were fixed in methanol-free 4% formaldehyde (Pierce) in PBS for 30 min, washed 3 times for 15 min in 0.1% Tween-20 PBS and transferred on a fresh slide containing Vectashield mounting medium with DAPI (Vectorlabs, Inc.). Cover slips were added to gently crack the spermathecae and release sperm. Samples in which the sperm nuclei were sufficiently diluted were then subjected to further analysis. Multiple-plane z-series were collected with a confocal microscope (SP5; Leica) and a 23× lens. Confocal microscope z-series were analyzed using image-analysis software (Volocity; Improvision Inc.). Stacked images were used to render 3D reconstructions of the sperm nuclei. Objects were defined on the basis of DAPI fluorescence intensity and by size, and were then measured for DAPI and GFP density (intensity/volume).

Embryo Fixation and Nuclear Staining

Sterile embryos were collected from crosses of b2Ppo males mated with WT virgin females and control embryos from crosses of WT males with b2Ppo females. Females were allowed to egg-lay 48 hrs post blood-feeding. The exochorion of up to 24 hrs old embryos was removed and embryos fixed essentially as described [36]. Fixed embryos were stored at −20°C in methanol. To stain nuclei, the exochorion was gently peeled off by submerging embryos on double side tape in methanol and gently stroking them out using a fine brush. Embryos were rehydrated in PBTA (1× PBS, 1% BSA, 0.05% Triton X-100, 0.02% Sodium Azide) for 15 min on a rotator. DNA was stained for 15 min in the dark with DAPI (1 μg/ml) and washed twice for 1 hour and once overnight with fresh PBTA avoiding unnecessary light exposure. Embryos were then mounted on slides and subjected to confocal analysis (SP5; Leica).

Immunohistochemistry

Rehydrated embryos were probed with mouse monoclonal anti γ-H2AX (Ser139 mouse monoclonal; Upstate Biotechnology; 1:200) to detect the phosphorylated form of histone H2AX. Alternatively embryos were probed with mouse monoclonal anti-GFP (Living Colors, JL-8; 1:200). Embryos were probed overnight at 4°C and then washed 3 times and once for one hour in PBTA. As secondary antibody we used goat anti-mouse IgG Alexa-532 conjugate (Molecular Probes; 1:500) to detect the phosphorylated form of histone H2AX. Embryos were then mounted on slides and subjected to confocal analysis (SP5; Leica).

Single Embryo Genotyping

Embryos were homogenized in 5 μl extraction buffer (10 mM Tris, pH 8.2; 1 mM EDTA; 25 mM NaCl) containing 200 μg/ml proteinase K (Sigma) and incubated for 1 h at 37°C followed by 10 min at 95°C. The whole extraction was then used in a 25 μl outer PCR reaction using the Phusion Hotstart DNA polymerase (Finnzymes). 0.5 μl of this PCR was used in an inner reaction with the same conditions: (35 sec at 98°C; 35 rounds of 1 sec at 98°C, 45 sec at 61°C, 30 sec at 72°C; and 5 min at 72°C). The nested primers used were: ST1F, GGGCAGTCATCCTAGTGTCG; ST2F, GAATCGAACCTCCTGGTCGTTGTA and ST7OR, CTITTTGTCGCACCGCA; STIR, GTAGCTGTCGAACCTTCCGG for the amplification of the Y chromosome. Primers mag-mdg1F, ATGTAGCAGTGTCAGCAGTTC; mag-mdg1OR, GCTTCTTGAGTTGAGCCAAG; mag-mdg1OR, CCGGTGTTTTGGTCTGGTCA were used to check for the presence of the Y chromosome [33]. Primers PpoIF, CGACTTAAAGAAGAAGGTA; PpoOF, GACCTGTACAAGTCCGGCTAGT; and PpoIF, CTTTGTTGAGGACCTGCCACGT; PpoOR, CITATACCAAAATGACTGCGCCCT amplify the I-Ppo1 open reading frame to check for presence of the transgene.

Supporting Information

Figure S1 Location of transgene integration sites and genomic rDNA repeats. Positions of insertions are shown as well as the 14 basepairs flanking the transformation constructs on each side (lower right panel). The structure of the rDNA repeat unit including the 3 ribosomal genes and the internal transcribed spacers (ITS) as well as a detailed view of the 28S rDNA gene around the I-Ppo1 recognition site is shown in the upper right panel. Primers RdFwd and RdRev were used to generate the 2kb probe for southern hybridization. Found at: doi:10.1371/journal.pgen.1000291.s001 (0.42 MB TIF)

Table S1 Outcome of crosses between transgenic b2Ppo and WT mosquitoes. Heterozygote b2Ppo2 males of generation 3 were crossed to WT females. As control b2Ppo2 heterozygote females of generation 3 were crossed to WT males. The total number of eggs laid and larvae hatched are shown for two consecutive egg depositions (Lay1 and Lay2). In addition larvae originating from control crosses were screened for the 3xP3-DrRed marker to determine the numbers of WT and transgenic offspring as indicated. Found at: doi:10.1371/journal.pgen.1000291.s002 (0.05 MB JPG)

Acknowledgments

We would like to thank Miriam Menichelli, Ann Hall and Kalle Magnusson for help. We thank Tony Nolan for proofreading the manuscript. We thank Austin Burt for encouragement and useful discussions.

Author Contributions

Conceived and designed the experiments: NW PAP. Performed the experiments: NW PAP. Analyzed the data: NW PAP. Contributed reagents/materials/analysis tools: PAP. Wrote the paper: NW AC. Initiated the project: NW. Supervised and inspired the project: AC.

References

1. World Health Organization (2005) World Malaria Report 2005: Roll Back Malaria. Geneva: WHO.
2. Kouyate B, Sie A, Ye M, De Allegri M, Muller O (2007) The great failure of malaria control in Africa: a district perspective from Burkina Faso. PLoS Med 4: e127.
3. Curris CT, Graves PM (1988) Methods for replacement of malaria vector populations. J Trop Med Hyg 91: 43–48.
4. Beatty BJ (2000) Genetic manipulation of vectors: A potential novel approach for control of vector-borne diseases. Proc Natl Acad Sci U S A 97: 10295–10297.
5. Carlson J, Olson K, Higgs S, Beatty B (1995) Molecular genetic manipulation of mosquito vectors. Annu Rev Entomol 40: 359–388.
6. Christophides GK (2005) Transgenic mosquitoes and malaria transmission. Cell Microbiol 7: 325–333.
7. O’Brocha DA, Handler AM (2008) Perspectives on the state of insect transgenesis. Adv Exp Med Biol 627: 1–18.
8. Chen CH, Huang H, Ward CM, Su JT, Schaeffer LV, et al. (2007) A synthetic maternal-effect selfish genetic element drives population replacement in Drosophila. Science 316: 597–600.
9. Thomas DD, Donnelly CA, Wood RJ, Alphey LS (2000) Insect population control using a dominant, repressible, lethal genetic system. Adv Exp Med Biol 627: 1–18.
10. Alphey L, Andersen M (2002) Dominant lethality and insect population control. Mol Biochem Parasitol 121: 173–178.
11. Sinkins SP, Gould F (2006) Gene drive systems for insect disease vectors. Nat Rev Genet 7: 427–435.
12. James AA (2005) Gene drive systems in mosquitoes: rules of the road. Trends Parasitol 21: 64–67.
13. Burt A (2003) Site-specific selfish genes as tools for the control and genetic engineering of natural populations. Proc Biol Sci 270: 921–928.
14. Cook PE, McMeniman CJ, ONeill SL (2008) Modifying insect population age structure to control vector-borne disease. Adv Exp Med Biol 627: 126–140.
15. Robinson SA, Franz G (2000) The Application of Transgenic Insect Technology in the Sterile Insect Technique. In: Handler AM, James A, eds. Insect Transgenesis: Methods and Applications CRC Press.
16. Catteruccia F, Benton JP, Crisanti A (2005) An Anopheles transgenic sexing strain for vector control. Nat Biotechnol 23: 1414–1417.
17. Horn C, Wimmer EA (2003) A transgene-based, embryo-specific lethality system for insect pest management. Nat Biotechnol 21: 64–70.
18. Windbichler N, Papadimos PA, Catteruccia F, Ranson H, Burt A, et al. (2007) Homing endonuclease mediated gene targeting in Anopheles gambiae cells and embryos. Nucleic Acids Res 35: 5922–5933.
19. Hamilton WD (1967) Extraordinary sex ratios. A sex-ratio theory for sex linkage and inbreeding has new implications in cytogenetics and entomology. Science 156: 477–488.
20. Hickey WA, Craig GB Jr. (1966) Genetic distortion of sex ratio in a mosquito, Aedes aegypti. Genetics 53: 1177–1196.
21. Wood RJ, Newton ME. (1991) Sex-ratio distortion caused by meiotic drive in mosquitoes. Am Nat 137: 379–391.
22. Newton ME, Wood RJ, Southern DI (1976) A cytogenetic analysis of meiotic drive in the mosquito, Aedes aegypti (L.). Genetica 46: 297–318.
23. Sweeney TL, Burt AR (1976) Sex Ratio Distortion Caused by Meiotic Drive in a Mosquito, Culex pipiens L. Genetica 80: 427–446.
24. Collins FH, Mendez MA, Rasmussen MO, McHaffey PC, Besansky NJ, et al. (1987) A ribosomal RNA gene probe differentiates member species of the Anopheles gambiae complex. Am J Trop Med Hyg 37: 37–41.
25. Collins FH, Paskewitz SM, Finney V (1989) Ribosomal RNA genes of the Anopheles gambiae species complex. Advances Disease Vector Res 6: 1–28.
26. Paskewitz SM, Collins FH (1990) Use of the polymerase chain reaction to identify mosquito species of the Anopheles gambiae complex. Med Vet Entomol 4: 367–373.
27. Ellison EL, Vogt VM (1993) Interaction of the intron-encoded mobility endonuclease I-PpoI with its target site. Mol Cell Biol 13: 7531–7539.
28. Sharakhova MV, Hammond MP, Lobo NF, Krzywinski J, Unger MF, et al. (2007) Update of the Anopheles gambiae FEST genome assembly. Genome Biol 8: R5.
29. Argast GM, Stephens KM, Emond MJ, Monnat RJ Jr. (1998) I-PpoI and I-CreI homing site sequence degeneracy determined by random mutagenesis and sequential in vitro enrichment. J Mol Biol 280: 343–353.
30. Benton J (2007) Transgenic sterile insect technique in Anopheles mosquitoes. London.
31. Braun RE, Behringer RR, Peschon JJ, Brinster RL, Palmeter RD (1989) Genetically haploid spermatids are phenotypically diploid. Nature 337: 373–376.
32. Jang JK, Sherizen DE, Bhagat R, Manheim EA, McKim KS (2003) Relationship of DNA double-strand breaks to synopsis in Drosophila. J Cell Sci 116: 3069–3077.
33. Kraywinski J, Nusskern DR, Kern MK, Besansky NJ (2004) Isolation and characterization of Y chromosome sequences from the African malaria mosquito Anopheles gambiae. Genetics 166: 1391–1392.
34. Horn C, Wimmer EA (2000) A versatile vector set for animal transgenesis. Dev Genes Evol 210: 630–637.
35. Lobo NF, Clayson JR, Fraser MJ, Kafatos FC, Collins FH (2006) High efficiency germline transformation of mosquitoes. Nat Protoc 1: 1312–1317.
36. Goltsy V, Hsiang W, Lanzaro G, Levine M (2004) Different combinations of gap repressors for common stripes in Anopheles and Drosophila embryos. Dev Biol 275: 435–446.