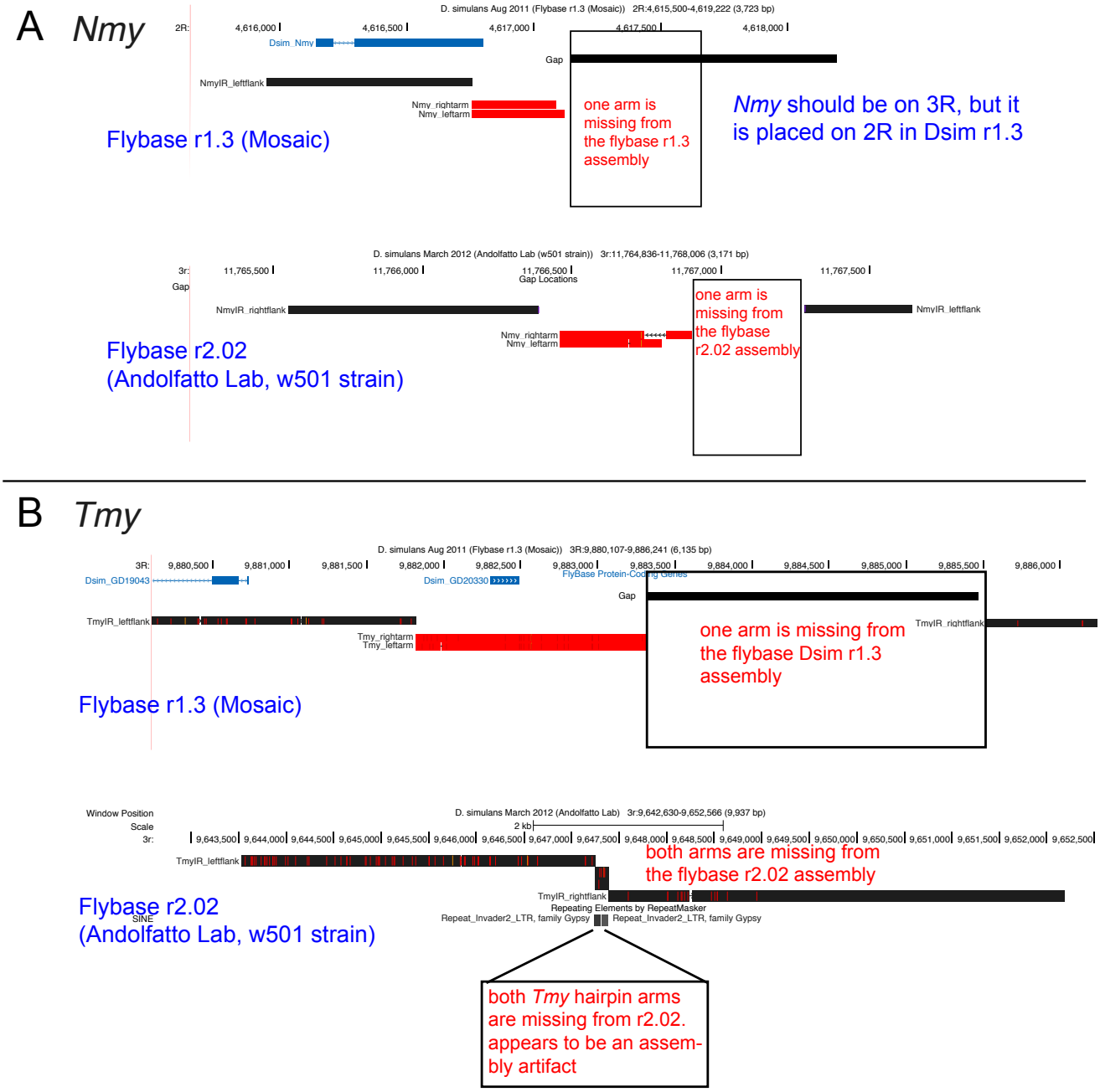

Figure S1. Related to Figures 1 and 2.
Assembly issues of Nmy and Tmy regions in available D. simulans genome versions.

(A) *Nmy* is genetically known to localize to chromosome 3R, but it is misplaced onto 2R in the mosaic *D.simulans* genome assembly r1.3. This version also contains a gap, so that only one hairpin arm of *Nmy* is contained in the assembly. Since we have biochemically shown that these siRNAs are produced from a hpRNA precursor, and that a *nmy* mutant deleted for one of hpRNA arms cannot produce siRNAs, we know that this represents an assembly artifact. In the updated assembly r2.02 from the w501 strain, *Nmy* is correctly placed at the known location on chromosome 3R, but it still contains a gap, such that only one hairpin arm is present in the assembly. (B) *Tmy* is genetically delimited by introgressions to an ~80 kb region on chromosome 3R. We recognized a small RNA producing locus in this region that includes perfect matches to some *Nmy* siRNAs. This region is gapped in Dsim r1.3, and the small RNA producing regions are missing from the r2.02. However, PacBio assembly (Figure 2) resolves both of these regions within a full contig, showing that both *Nmy* and *Tmy* are hpRNAs.

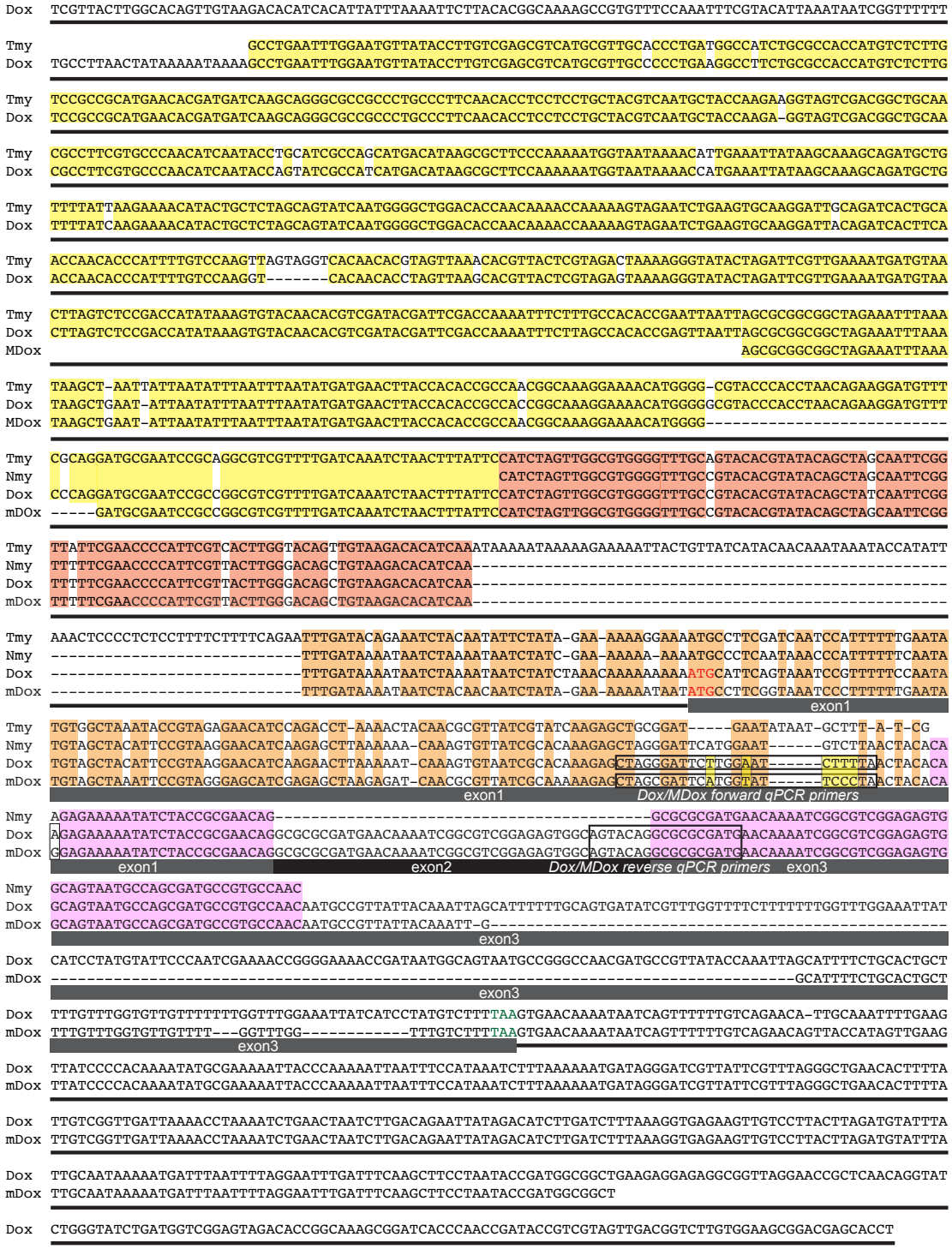

Figure S2, Related to Figures 2-4.
Alignment of the complementary hairpin arms of *Tmy* and *Nmy* with the transcribed strands of *Dox* and *MDox*.

Regions are color-coded as in Figure 4, according to whether there is pairing only between *Tmy* and *Dox* (yellow), pairing amongst all four (orange) or pairing between *Nmy* and *Dox*/*Mdox* (purple). The positions of *Dox*/*MDox* qPCR primers are noted in boxes. We were able to design primers in exon 1 with at least 6 mismatches between *Dox* and *mDox*, and especially that exhibit at least two mismatches within the last 5 nt of the primers. In addition, there is a mismatched nucleotide within the amplicon that we used to verify on-target amplification of qPCR products by sequencing.

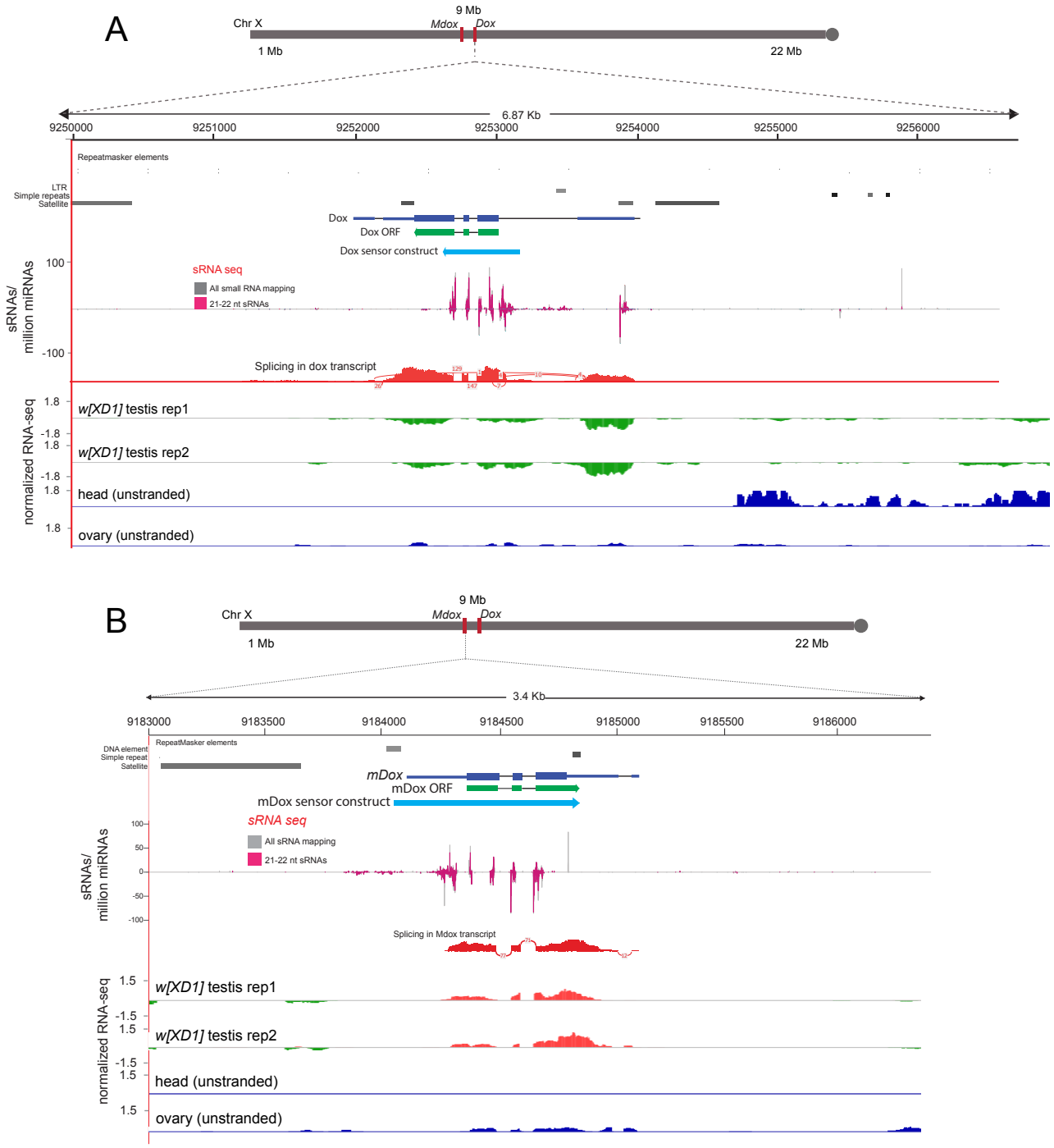

Figure S3, Related to Figure 4.
Genomics of the Dox and MDox loci, which are closely linked on the X chromosome.

(A) Summary of RNA-seq and *Nmy*/*Tmy* small RNA matching to the *Dox* locus. *Dox* is much higher expressed in testis compared to ovary, and is not detected in head. The *Dox* transcript is extensively targeted by siRNAs. The region used in the *Dox* sensor is indicated. (B) Summary of RNA-seq and *Nmy*/*Tmy* small RNA matching to the *MDox* locus. *MDox* is much higher expressed in testis compared to ovary, and is not detected in head. The *MDox* transcript is extensively targeted by siRNAs. The region used in the *MDox* sensor is indicated.

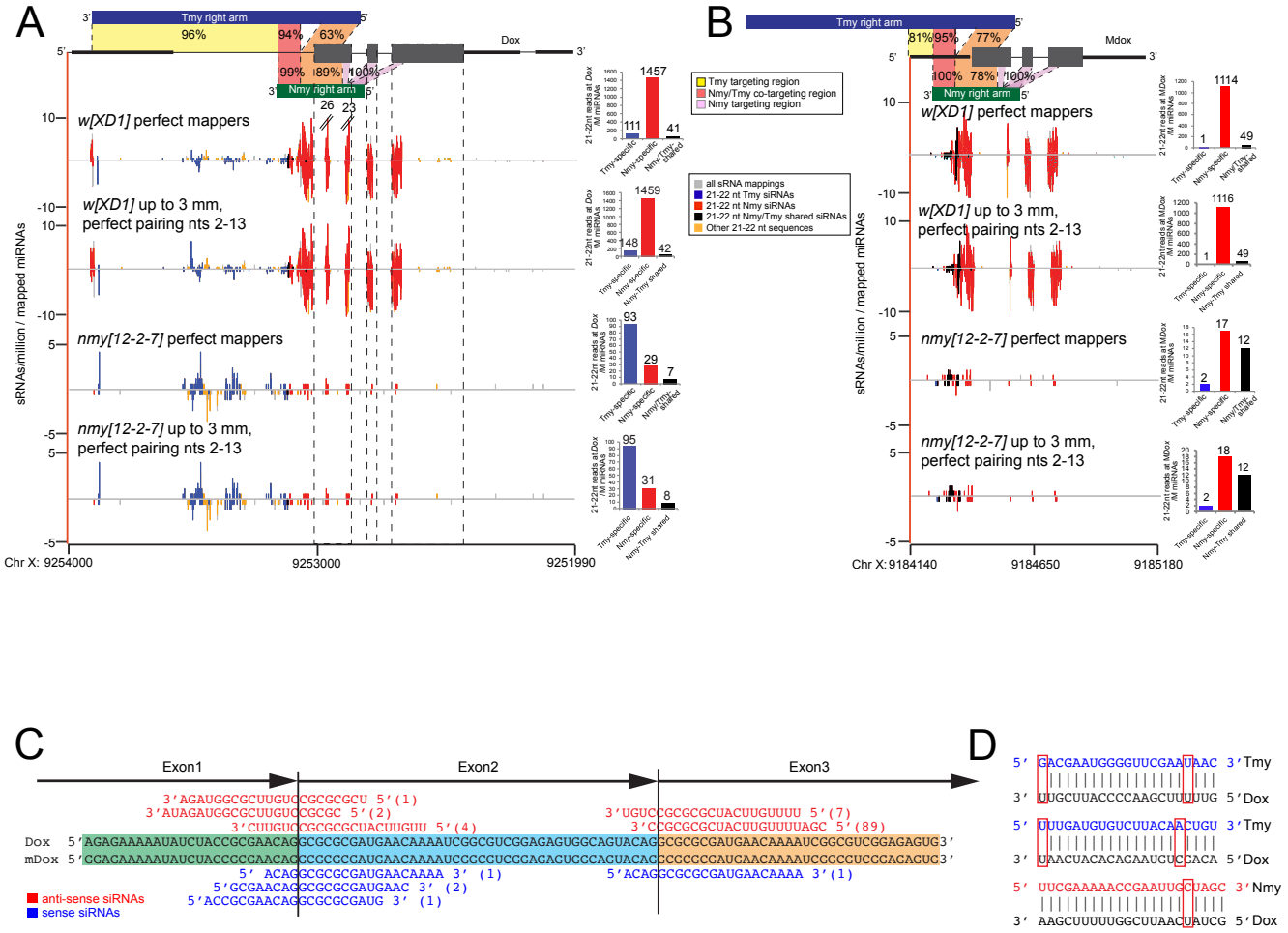

Figure S4. Related to Figure 4.
Comparison of perfect and imperfect Nmy/Tmy siRNA matching to *Dox*/*MDox* targets.

(A) Allowing restricted mismatches, with required perfect pairing in an extended seed region (from siRNA nts 2-13) recovered modest increase in siRNA mapping to the *Dox* and (B) *MDox* loci for *w[XD1]* and *nmy[12-2-7]*, indicating that a strong majority of siRNAs match perfectly to *Dox* and *MDox*. (C) siRNA mapping to *Dox* and *MDox* splice junctions reveal target complementary siRNAs at exon junctions, which are omitted when mapped to *Dox*/*MDox* DNA sequence. However, mapping to *Dox*/*MDox* DNA sequence highlights a preponderance of siRNAs mapping only to exons, evident from the homology relationship that *Nmy* hpRNA loci contains a retroposed copy of the *Dox*/*MDox* target. (D) Examples of siRNA complementary targeting with restricted mismatches allowed at nts 1 and 14 and above, preserving and extended siRNA seed pairing through nts 2-13.

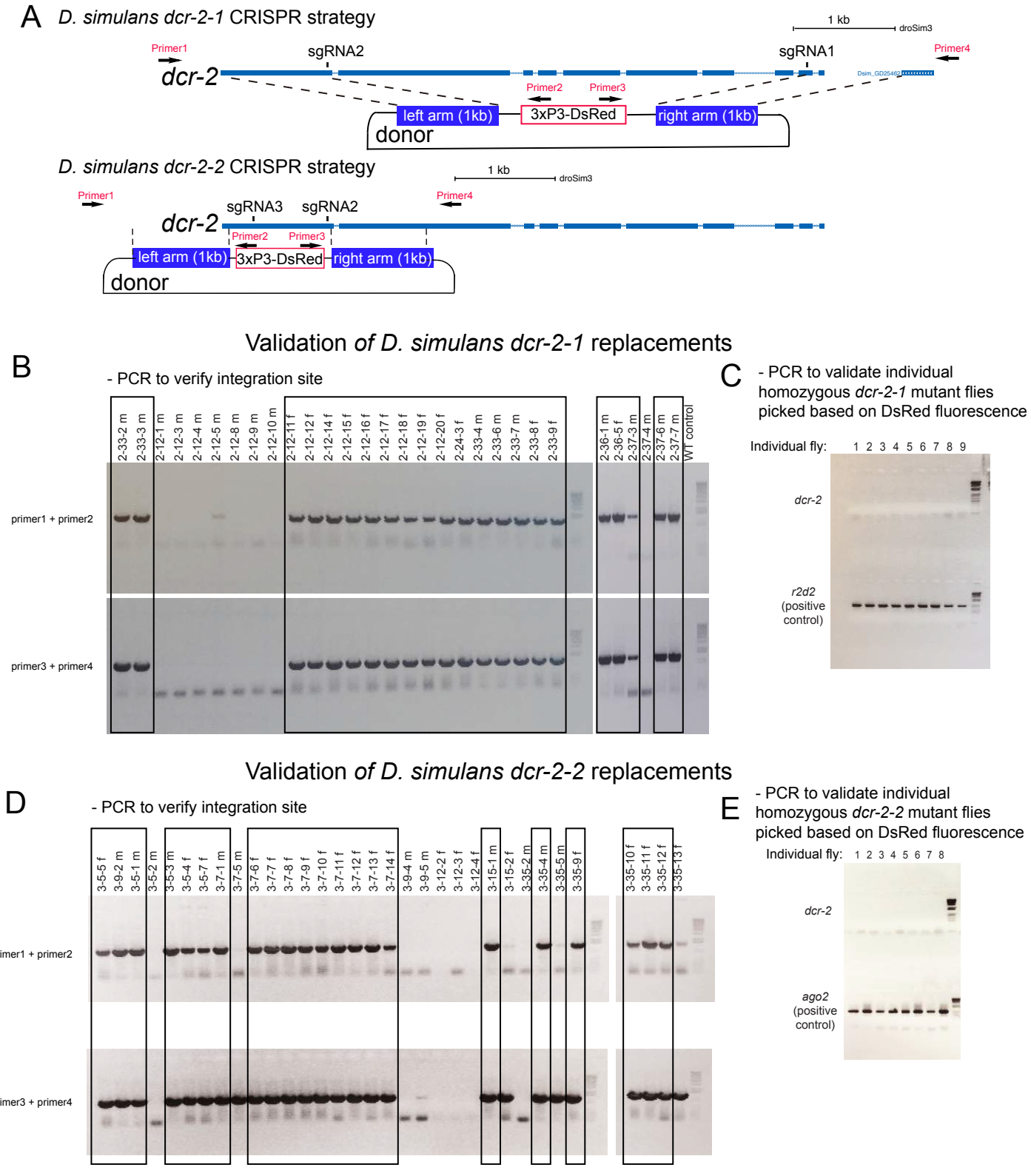

Figure S5. Related to Figure 4. *Dcr-2* CRISPR mutagenesis strategy and validation.

(A) Location of *dcr-2* sgRNAs, homology donor arms and 3xP3-DsRed reporter used for mutant selection to generate two different deletion alleles of *dcr-2* (*dcr-2-1* and *dcr-2-2*). (B-C) Selection and validation of *dcr-2-1* mutants. (B) Candidate DsRed positive flies were genotyped for marker integration at the *dcr2* locus. Boxed PCR tests indicate positive hybrid amplification on both left and right flanks. (C) Genotyping of individual candidate homozygous *dcr-2* mutant animals for deletion of *dcr-2* DNA; amplification of *r2d2* DNA serves as a positive control. (D-E) Selection and validation of *dcr-2-2* mutants. (D) Candidate DsRed founders were genotyped for marker integration at the *dcr-2* locus. Boxed PCR tests indicate positive hybrid amplification on both left and right flanks. Note that some animals only validated on one flank and were not considered further, although they likely contained a disruption of the *dcr-2* gene. (E) Genotyping of individual candidate homozygous *dcr-2* mutant animals for deletion of *dcr-2* DNA; amplification of *ago2* DNA serves as a positive control. All primer sequences are listed in Table S2.

D. simulans AGO2 CRISPR strategy

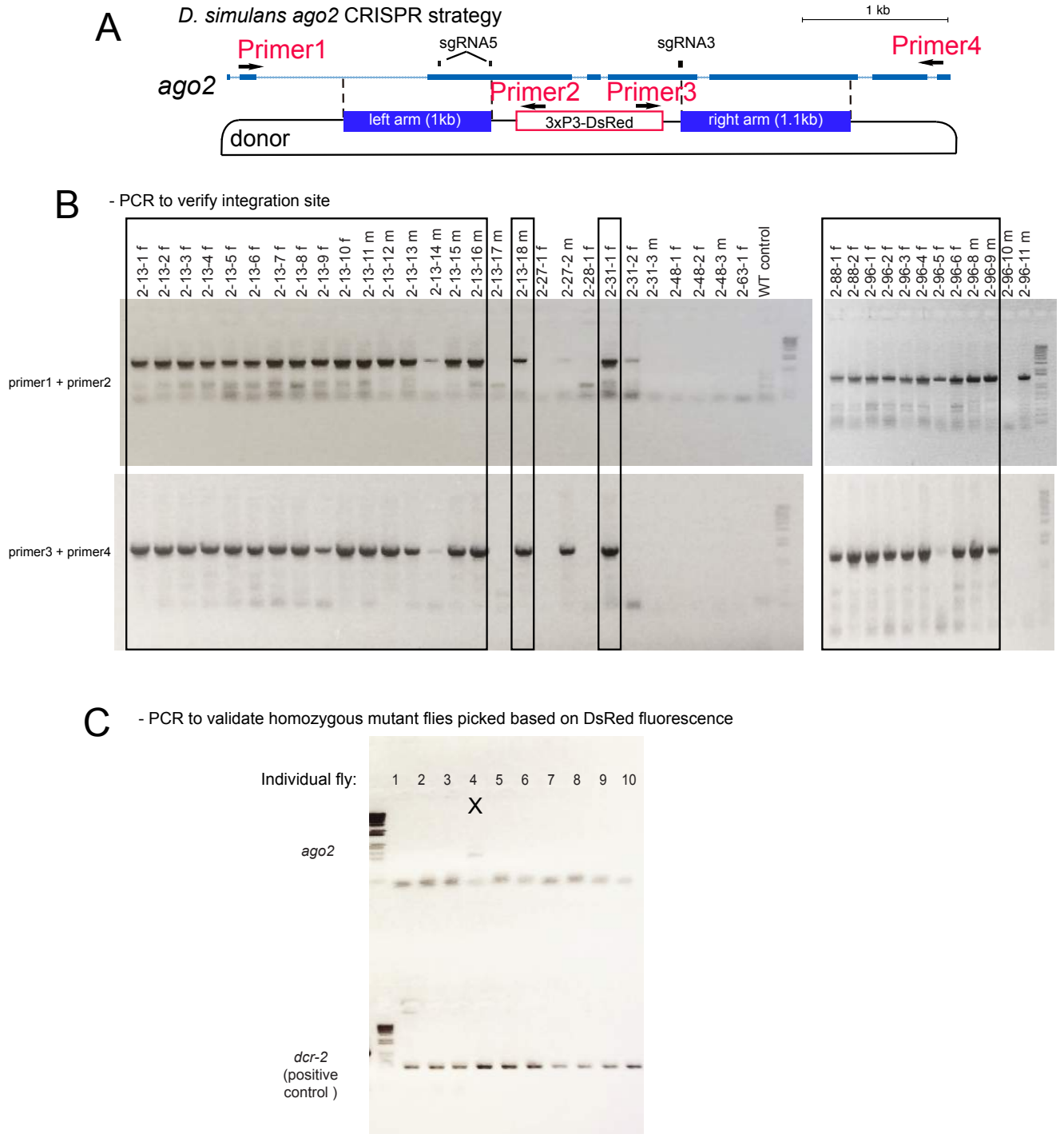

Figure S6. Related to Figure 4.
***Ago2* CRISPR mutagenesis strategy and validation.**

(A) Location of *ago2* sgRNAs, homology donor arms and 3xP3-DsRed reporter used for mutant selection. (B) Candidate DsRed positive mutants were genotyped for marker integration at the *ago2* locus. Boxed PCR tests indicate positive hybrid amplification on both left and right flanks. Note that some animals only validated on one flank and were not considered further, although they likely contained a disruption of the *ago2* gene. (C) Genotyping of individual candidate homozygous *ago2* mutant animals for deletion of *ago2* DNA; amplification of *dcr-2* DNA serves as a positive control. Note that fluorescence is not entirely reliable for genotyping, since lane 4 indicates a false positive (X) heterozygote with detection of wildtype *ago2* DNA. All primer sequences are listed in Table S2.

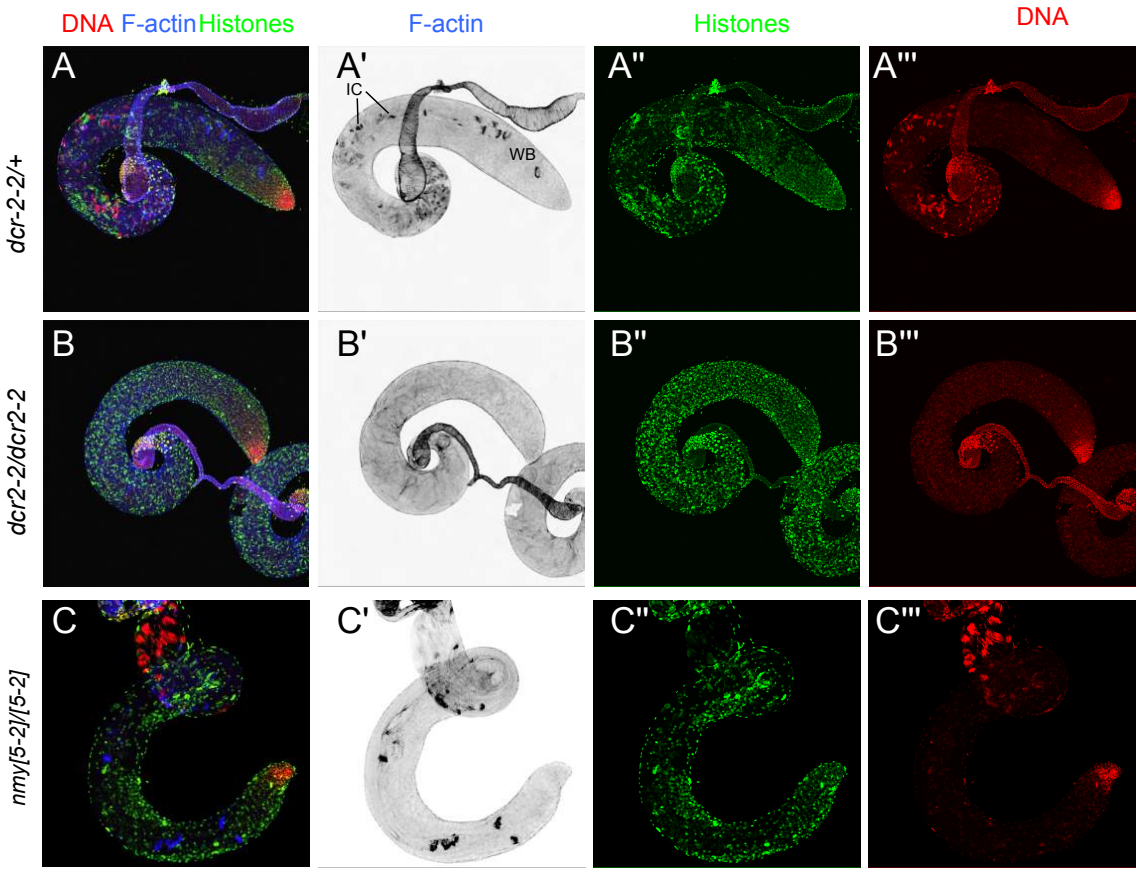

Figure S7. Related to Figure 6.
Cytological analysis of *dcr-2-2* mutants and *nmy[5-2]* mutants.

(A-C) Whole testes stained for DNA (red), histones (green) and F-actin (blue), showing that the normal pattern of spermatogenesis in control (A, *dcr-2-2* heterozygote) is severely disrupted in *dcr-2-2* homozygote (B) but not in *nmy[5-2]* homozygote (C) that exhibits severe sex ratio bias. F-actin staining highlights individualization complexes (IC) that separate spermatids into individual cells to form wastebags (WB) These actin structures found in control (A') are completely absent in *dcr-2-2* mutant (B') but are present in *nmy[5-2]* mutant (C') testis. In addition, the histone-to-protamine transition of post-meiotic spermatid nuclei is evident in control testis (A''), but histones remain associated throughout in *dcr-2-2* testis (B''). This transition occurs normally in *nmy* mutant testis (C'').

Table S1, Related to Figure 1, 2, 3, S3 and S4.
Small RNA and RNA-seq libraries used in this study.
This table depicts the details of public and new small RNA and RNA-seq datasets analyzed in this study.
For each dataset, its tissue origin and mapping statistics are provided.

Mapping statistics for small RNA libraries used in this study.

acc/SRA_acc	tissue	description	total mapped reads
M023	head	head	38990369
SRR1205790	male_body	male_body	37988067
M025	embryo	embryo	79194312
M053	female_body	female_body	123490687
V044	embryo	embryo	117360240
SRR553485	ovary	Chicharo_3 day-old_ovaries	394537604
SRR553486	ovary	Makindu_3 day-old_ovaries	474328738
SRR553487	egg	NRT_0-2 hours_eggs	254889630
SRR553488	egg	RT_0-2 hours_eggs	203151228
SRR618934	ovary	dsim_w501_ovaries	275825212
SRR618935	body	F1_dmel-dsim_interspecies_body	141315165
SRR902008	ovary	ovary	198907535
SRR902009	testis	testes	100562829
small RNA datasets produced for this study			
wXD1_1_sRNA	testes	testes - wildtype	19079372
wXD1_2_sRNA	testes	testes - wildtype	5869386
12-2-7_1_sRNA	testes	testes - nmy mutant	17694739
12-2-7_2_sRNA	testes	testes - nmy mutant	11226479

Mapping statistics for RNA seq libraries used in this study.

Sample	Tissue	Total reads	mapped reads	Type	map %	Non primar	Unmapped	non-unique	Unique	Read 1	Read 2	Reads map	Reads map	Non-splice	Splice reads	Proper pairs
SRR1107945	head	6130129	5416328	Paired, unstr	88.36	347285	713801	124082	4944961	2529102	2415859	2479528	2465433	4651085	293876	4338926
SRR330570	ovary	81046293	65750134	Paired, unstr	81.13	27035605	15296159	12070538	26643991	13521221	13122770	13241693	13402298	18725432	7918559	22866244
wXD1_1	testis	29917669	29240366	Paired, stran	97.74	3998809	677303	2245876	22995681	11510649	11485032	11503335	11492346	20553691	2441990	22621452
wXD1_2	testis	33177913	32393175	Paired, stran	97.63	4274447	784738	2400445	25718283	12882002	12836281	12868703	12849580	22983207	2735076	24724214

Table S2, Related to STAR methods. **Oligonucleotides used in this study.** This table lists all primers used for cloning and for genrating RNA-seq libraries, and oligos used as probes.		
Primers for pDCC6-sgRNA construct		
ago2 sgRNA3:GTAGTTGCGACTGTGGAAGT (AGG)	Fwd	cttcGTAGTTGCGACTGTGGAAGT
	Rev	aaacACTTCCACAGTCGCAACTAC
ago2 sgRNA4:GCTCATTTGGTAGCTGCTCG (CGG)	Fwd	cttcGCTCATTTGGTAGCTGCTCG
	Rev	aaacCGAGCAGCTACCAAATGAGC
ago2 sgRNA5: GAAAACACAAGGCCAGGCTC (GGG)	Fwd	cttcGAAAACACAAGGCCAGGCTC
	Rev	aaacGAGCCTGGCCTTGTGTTTTC
dcr-2 sgRNA1:GGCATTGTATACCTGCCCAC (AGG)	Fwd	cttcGGCATTGTATACCTGCCCAC
	Rev	aaacGTGGGCAGGTATACAATGCC
dcr-2 sgRNA2:GGTGATAGCCGATACCCTTG (AGG)	Fwd	cttcGGTGATAGCCGATACCCTTG
	Rev	aaacCAAGGGTATCGGCTATCACC
dcr-2 sgRNA3:GACGTTGGGGGATGTTCTGG (AGG)	Fwd	cttcGACGTTGGGGGATGTTCTGG
	Rev	aaacCCAGAACATCCCCCAACGTC
Primers for pHD-DsRed HRD construct		
ago2 sgRNA3 and sgRNA5	Left arm Fwd	GAGGAATTCGACGTGGTTGTATAGAACCTATG
	Left arm Rev	GACCCGCGGCCTGGCCTTGTGTTTTCTGTCC
	Right arm Fwd	GAGGGCGCGCCTCCACAGTCGCAACTACCCATTG
	Right arm Rev	GAACTCGAGCCAAAGTTACTGTGTACATGTC
dcr-2 sgRNA1 and sgRNA2	Left arm Fwd	GAGGAATTCGCTTGGCCTGGTTCTTATTGC
	Left arm Rev	GACCCGCGGTTGAGGCGCTATTGGGCGTG
	Right arm Fwd	GAGACTAGTGGCAGGTATACAATGCCATTGC
	Right arm Rev	GAAGGCGCGCCAGCCAGACGTGATGATGTC
dcr-2 sgRNA2 and sgRNA3	Left arm Fwd	GAGGAATTCCGGGGTAACCCCAAATGTTAC
	Left arm Rev	GACCCGCGGCCAAAGACGTTGGGGGATGTTC
	Right arm Fwd	GAGACTAGTGGGTATCGGCTATCACCTTG
	Right arm Rev	GAAGGCGCGCCGATGTCGAGGAGCCGAAATC
Primers for genotyping		
Checking deletion		
ago2[DsRed]	ago2 sgRNA3 target Fwd	ggaatattcgcgacgcttccttg
	ago2 sgRNA3 target Rev	gatggtcggatccaggttgtgc
	ago2 sgRNA5 target Fwd	gagatacaaaatattttgtatcg
	ago2 sgRNA5 target Rev	ggtattggccttggctctggg
dcr-2[DsRed]	dcr2 sgRNA1 target Fwd	cagctaaactatgcaagagcc
	dcr2 sgRNA1 target Rev	cgatttcatcactccactttc
	dcr2 sgRNA2 target Fwd	ccgcttcgtggagggatgtcc
	dcr2 sgRNA2 target Fwd	atcggcttcctcgagcagaatg
	dcr2 sgRNA3 target Fwd	gttcagccgacccgctggac
	dcr2 sgRNA3 target Fwd	ttaggcgtcgcatttgcttagc
Checking insertion of DsRed		
Primers on 3XP3-DsRed	3xP3 Rev	ATTGTCGCTCCGTAGACGAAGC
	UniSV40 seq fwd	GTTAACTTGTTTATTGCAGCTTATAATGG
ago2[DsRed]	AGO2 insertion site left fwd	CATTCTCGGCGTATGTAAAAAAC
	AGO2 insertion site right rev	GGCCGTCGCTCACGCCATCTCGG
dcr-2[DsRed]-1	Dcr2-1 insertion site left fwd	GGAAACTCGAATGGACTTCTAG
	Dcr2-1 insertion site right rev	CTTCTACTCTCTGTTCTTCGTGC
dcr-2[DsRed]-2	Dcr2-2 insertion site left fwd	CGACAGCGCAGCATCAATAAGCC
	Dcr2-2 insertion site right rev	GCTGCCTTTCAATGGAACTG
Primers for cloning hpRNA constructs		
Nmy/nmy_F	ATAAGAATGCGGCCGCACGACACCTCTGCCAAGTAG	
Nmy/nmy_R	CTAGTCTAGAATTTTTAGTTCAATTTTTATTAACCAATTATAC	
Tmy_F	ATAAGAATGCGGCCGCGCCTGAATTTGGAATGTTATACC	
Tmy_Loop_R	CCGCTCGAGACGGTATAACGGCATCCTTG	
Tmy_Loop_F	CCGCTCGAGTAGTTCATCATATTAGCGGTAGCGATTTTTCTGA	
Tmy_R	GCTCTAGAGCCTGAATTTGGAATGTTATACC	
Primers for cloning sensor constructs.		
Dox-sensor-F	AAAAGGAAAAGCGGCCGCCATCTAGTTGGCGTGGGGTT	
Dox-sensor-R	CCGCTCGAGGTTGGCACGGCATCGCTGG	
mDox-sensor-F	GCGGCCGCACTTAGTCTCCGACCATATA	
mDox-sensor-R	GGACTAGTAAAAGACAAACCAAACCAAA	
qPCR primers		
Dox-qPCR-F	CTAGGGATTCTTGGAATCTTTTA	
mDox-qPCR-F	CTAGCGATTCATGGTATTCCCTA	
Dox/mDox-qPCR-R	CATCGCGCGCCTGTACT	
Rpl32 fw	CCGCTTCAAGGGACAGTATC	
Rpl32 rev	ACGTTGTGCACCAGGAACTT	
probes for Northern blotting_Nmy		
Nmy_5388	ATGTGTCTTACAGCTGTCCCA	
Nmy_2307	GTGTCTTACAGCTGTCCCAAG	
Nmy_2210	CTCTCCGACGCCGATTTTGTT	
Nmy_2065	TTGATGTGTCTTACAGCTGTC	
Nmy_1829	CTCTCCGACGCCGATTTTGT	
Nmy_1684	TGTGTCTTACAGCTGTCCCAA	
Nmy_630	GCTGTATACGTGTACGGCAAAC	
Nmy;Tmy_551	ACACGTATACAGCTAGCAATT	
Nmy;Tmy_444	GTACACGTATACAGCTAGCAA	
probes for Northern blotting_Tmy specific		
Tmy_989	GTTATTCGAACCCCATTCGTCA	
Tmy_514	ATGTGTCTTACAACTGTACCA	
Tmy_13081	ATGCCTTCGATCAATCCATTT	
Tmy_13070	ATACCGTAGAGAACATCCAGA	
Tmy_528	AACTCCCCTCTCCTTTTCTTT	
oligos for small RNA library cloning		
illumina_RT_primer	GCCTTGGCACCCGAGAATTCCA	
illumina_PCR_FW	AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA	
illumina_PCR_REV	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	
3' linker	5' phos-TGGAATTCTCGGGTGCCAAGG-ddC 3'	
5' linker	5' ddT- rGrUrUrCrArGrArGrUrUrCrUrArCrArGrUrCrCrGrArCrGrArUrC 3'	
	standard illumina barcode primers were used for multiplexing	