

# Homology directed repair is unaffected by the absence of siRNAs in *Drosophila melanogaster*

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Received November 02, 2015; Revised June 13, 2016; Accepted June 14, 2016

## ABSTRACT

Small interfering RNAs (siRNAs) defend the organism against harmful transcripts from exogenous (e.g. viral) or endogenous (e.g. transposons) sources. Recent publications describe the production of siRNAs induced by DNA double-strand breaks (DSB) in *Neurospora crassa*, *Arabidopsis thaliana*, *Drosophila melanogaster* and human cells, which suggests a conserved function. A current hypothesis is that break-induced small RNAs ensure efficient homologous recombination (HR). However, biogenesis of siRNAs is often intertwined with other small RNA species, such as microRNAs (miRNAs), which complicates interpretation of experimental results. In *Drosophila*, siRNAs are produced by Dcr-2 while miRNAs are processed by Dcr-1. Thus, it is possible to probe siRNA function without miRNA deregulation. We therefore examined DNA double-strand break repair after perturbation of siRNA biogenesis in cultured *Drosophila* cells as well as mutant flies. Our assays comprised reporters for the single-strand annealing pathway, homologous recombination and sensitivity to the DSB-inducing drug camptothecin. We could not detect any repair defects caused by the lack of siRNAs derived from the broken DNA locus. Since production of these siRNAs depends on local transcription, they may thus participate in RNA metabolism—an established function of siRNAs—rather than DNA repair.

## INTRODUCTION

Genomes are constantly challenged by intrinsic and extrinsic damaging agents or processes; appropriate repair is thus essential. DNA double-strand breaks (DSBs) are particularly harmful lesions because they interrupt the continuity of genetic information. They arise physiologically due to replication fork stalling and collapse in S-phase of the mitotic cell cycle. Furthermore, the essential cross-overs

during the meiotic cell cycle are initiated by deliberately induced DNA DSBs. Finally, the rearrangement of immunoglobulin and T-cell receptor variable regions is triggered by DSB induction. Broken ends of DNA can be religated by the non-homologous end-joining (NHEJ) or the microhomology-mediated end-joining (MMEJ) pathways. Such events may lead to sequence losses and are thus mutagenic. Alternatively, homologous recombination (HR) can repair the break using the sister chromatid as template, but it is accordingly limited to the late S and G2 phases of the cell cycle.

Several pathways can mediate homologous recombination. These include gene conversion (GC), double Holliday junctions (dHJ), synthesis-dependent strand annealing (SDSA) and break-induced replication (BIR) (1). A special situation occurs if the DNA DSB arises between directly repeated sequences. In this case, the single-strand annealing (SSA) pathway can lead to the deletion of one copy of the direct repeat, thus shortening the genomic sequence. A common step in all HR events is the initial processing by the Mre11-Rad50-Nbs1 complex (MRN) together with the nuclease CtIP. This step is followed by further 3'-to-5' resection via exonuclease-1 (Exo-1); the downstream genetic requirements then differ between the individual HR pathways.

In recent years, the discovery of DNA DSB-derived siRNAs in plants as well as cultured human and *Drosophila* cells has raised the question whether small RNA mediated processes may influence the repair of DNA DSBs. Assays that report the activity of the SSA pathway in plants and homologous recombination in human cells showed decreased, but not abolished, repair activity when small RNA biogenesis factors were depleted (2,3). In addition, a physical interaction between the human siRNA effector protein Ago2 and the Rad51 recombinase has been described (3). Repair of damaged DNA follows different kinetics in the absence of locus-derived small RNAs (4), since the repair-associated  $\gamma$ -H2Ax foci persist for a longer time. On the other hand, generation of DSB-derived siRNAs only occurred if the break was located within a transcribed region in *Drosophila* (5). Furthermore, the phenotype of flies with mutations in the

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siRNA pathway is rather mild (6,7), while miRNA factor mutant flies are not viable (6,7).

Since the biogenesis enzymes for DSB-derived siRNAs usually also process precursors for other small RNAs, in particular miRNAs, it is challenging to conceive experiments that are not at risk of indirect effects via the miRNA pathway. Indeed, a recently published study concludes that the DSB-repair defect detected in cultured human cells depleted of Dicer is due to secondary effects on cell cycle progression and can be rescued by transfection of a let-7 miRNA mimic (8). Results in *Neurospora crassa*, on the other hand, have demonstrated that mutants of the Argonaute-protein Qde2 are hypersensitive to DNA damaging agents (9) and that DNA damage is necessary to trigger the transposon-defense mechanism of quelling in this organism (10). This required the DNA-dependent and RNA-dependent RNA polymerase activities of Qde1, which lacks homologs in both *Drosophila* and vertebrates.

In *Drosophila*, Dicer-1 removes the loop of pre-miRNAs to generate the miRNA/miRNA\* biogenesis intermediate. Double-stranded RNA, on the other hand, is nucleolytically processed into siRNAs by the paralog Dicer-2. Although Dicer-2 also processes endogenous precursors of the long hairpin derived endo-siRNAs, the *Drosophila* system allows examining the effects of siRNA removal without a major disturbance of the miRNA pathway. We therefore developed an SSA reporter as well as a homologous recombination assay based on a GFP knock-in strategy (11) for *Drosophila*. This allowed us to test the requirement of the siRNA pathway for repair efficiency. Neither in cultured cells depleted for siRNA biogenesis components nor in *dcr-2* mutant flies, an influence of siRNA factors on HR efficiency was observed. Finally, the challenge of *dcr-2* or *ago2* mutant flies with the DSB-inducing drug camptothecin did not show any hypersensitivity. We therefore hypothesize that the transcription dependent, break-derived siRNAs of *Drosophila* are part of an mRNA surveillance phenomenon that is invoked as a consequence of the perturbation of mRNA biogenesis caused by a DNA DSB within the transcribed region.

## MATERIALS AND METHODS

### Molecular biology

Tagging of the *act5C* locus with GFP via polymerase chain reaction (PCR) products or a targeting vector was performed as previously described (11). The SSA assay stable reporter cell line as well as DSB induction by *I-SceI* transfection was also previously published (12). To generate a  $\Delta lig4$  line in the SSA reporter background, cells were treated with a sgRNA against the *lig4* ORF with the targeting sequence 5'-GCCAGCACGATCAAGTTCC-3'. Subsequently a clonal selection was performed and a clone recovered where a deletion of ~0.8 kb removed the 5' UTR and the first 30 nt of the *lig4* ORF.

### Cell culture and transfection

Cells were cultured in standard Schneider's medium supplemented with 10% fetal bovine serum (FBS) (both Bio&Sell, Nürnberg/Germany). Cells were seeded at a density of 0.5

(for the HR assay using the short homology HR donor) or  $1 \times 10^6$  cells/ml (for all other experiments). Transfections were performed with Fugene HD (Promega) according to the manufacturer's instructions. For RNAi cells were treated with dsRNA (sequences published in (5,13–14) or described in Supplementary Table S1) at a final concentration of 2–10 ng/ $\mu$ l. For our SSA reporter cells were split 1:3 four days after induction of RNAi and transfected with pKF259 at a final concentration of 0.5 ng/ $\mu$ l (for introduction of the *I-SceI* nuclease). To assess transfection efficiencies, wildtype S2-cells were treated as described above but transfected with pKF63 (15) that encodes GFP expressed from the ubiquitin promoter. Readout by flow cytometry was performed five days post transfection.

For the HR assay with a donor molecule *in trans*, wildtype cells with stable Cas9 expression were seeded at a concentration of  $0.5\text{--}1 \times 10^6$  cells/ml and treated with dsRNA for RNAi. Two days after induction of RNAi, cells were transfected either with sgRNA/HR donor (0.75 ng/ $\mu$ l each) for tagging of *act5C* or with pKF63 (1.5 ng/ $\mu$ l) to assess transfection efficiencies. Cells were grown for an additional five days before measurement. For quantification of the percentage of GFP positive cells a Becton Dickinson FACSCalibur flow cytometer equipped with a 96-well plate autosampler was used. Data analysis was performed using flowing software version 2.5.1 (<http://www.flowingsoftware.com/>).

### Deep sequencing and data analysis

RNA extraction, library preparation and data analysis were performed as described previously (16). The sequences have been deposited at the European Nucleotide Archive (ENA) under the accession number PRJEB12939.

### Fly strains and husbandry

The fly strains carrying the *dcr-2* null allele (*w*; *dcr-2*[L811fsX]/*CyO*), the *dcr-2* catalytically inactive allele (*w*; *dcr-2*[G31R]/*CyO*) and the fly strain with the *dcr-2* rescue transgene *y,w,eyFLP*; *FRT42Ddcr-2*[L811fsX]; *P*{*w*<sup>+</sup>,*Dcr-2*} are described in (6). Transgenic flies carrying the SSA reporter and the *I-SceI* transgene were generated by PhiC31 recombinase mediated integration into *attP* sites 19E7 and 2A3, respectively; injections were performed by Rainbow Transgenics (Camrillo/CA, USA). The transgenes and *dcr-2* alleles were crossed together with standard genetic approaches.

For analysis of CPT sensitivity in absence of *dcr-2* or *ago2*, mutant strains with the reporter background were crossed to yield either homozygous or transheterozygous mutants. In addition to the fly strains mentioned above, we tested also the *dcr-2* mutant *w*[1118]; *PBac*{*w*[+*mC*] = *WH*}*dcr-2*[f06544]/*CyO* as well as the deficiency strain *y*[1] *w*[1118]/*Dp*(*I*; *Y*)*y*[+]; *Df*(2R)*ED3385*, *P*{*w*[+*mW*.*Scer*\*FRT.hs3*] = 3'.*RS5*+3.3'}*ED3385*/*CyO*.

We also investigated the CPT sensitivity in the absence of Ago2 by crossing the strain *w*[1118]; *AGO2*[321]/*TM3*, *Sb*[1], carrying an *ago2* loss of function allele to the *ago2* point mutant *yw*; *ago2*[V966M].

F0 females were kept on fly food coated with different concentrations of CPT (Sigma) or the respective DMSO

control during four days after eclosion. The cross was performed on fly food treated with different concentrations of CPT (or the respective control). After 7 days, flies were transferred to freshly treated fly food for additional 7 days. *dcr-2* proficient and *dcr-2* deficient progeny was counted. For the rescue experiment, *dcr-2* null mutants were crossed to flies carrying the *dcr-2* rescue transgene ( $\gamma, w, eyFLP$ ; *FRT42Ddcr-2*[*L811fsX*]; *P*{*w*<sup>+</sup>, *Dcr-2*}) and treated as described above. To evaluate the CPT sensitivity of a corresponding wildtype, flies carrying a *dcr-2* mutant allele were first backcrossed with *w*<sup>1118</sup>. The resulting offspring with a wildtype second chromosome and the balancer *CyO* was then used for the cross and CPT sensitivity assay as described above.

### Western blotting

A total of  $3 \times 10^6$  cells were treated with 3 ng/ $\mu$ l dsRNA and harvested for protein isolation after five days. Harvested cells were washed twice with 1x phosphate buffered saline (PBS) and lysed by resuspension in 1x PBS supplemented with 1x Proteinase Inhibitor without EDTA (Roche) and 1% Tween. Resuspended cells were frozen in liquid nitrogen, incubated at room temperature until almost complete thawing and then transferred to ice for 30 min. After a centrifugation step (16000 x g, 4°C, 15 min) supernatant was mixed with SDS-loading buffer and comparable amounts of protein were applied to a 12% SDS-gel.

For fly extraction individual flies were anesthetized, ground in 75  $\mu$ l lysis buffer (30 mM HEPES, 100 mM KOAc, 2 mM MgOAc, 1 mM DTT, 1% triton-X and 1x proteinase inhibitor cocktail (Roche)) and incubated on ice for 30 min. After centrifugation (16000 xg, 4°C, 15 min) the supernatant was mixed with SDS-loading buffer and 24  $\mu$ g of total protein was applied to a 12% SDS-gel. For Western blotting, the monoclonal B-2 antibody was used (sc-9996, Santa Cruz) for detection of GFP. For loading control the monoclonal anti beta-Tubulin (E7) antibody from the Developmental Studies Hybridoma Bank (DSHB) was used. Chemiluminescence images were recorded with a Fuji LAS-3000 mini system and quantification of band intensity was performed using ImageJ.

### Fluorescence microscopy

Fluorescence microscopy was performed on a Leica MZI6FA fluorescence stereomicroscope. Images were taken with a Leica DFC 320 camera at a fixed magnification (99.8x). Acquisition was done using Leica DFC Twain camera software with maximum gain setting and an exposure time of 2.9 s.

## RESULTS

### A reporter system to analyze DNA double-strand break repair in *Drosophila*

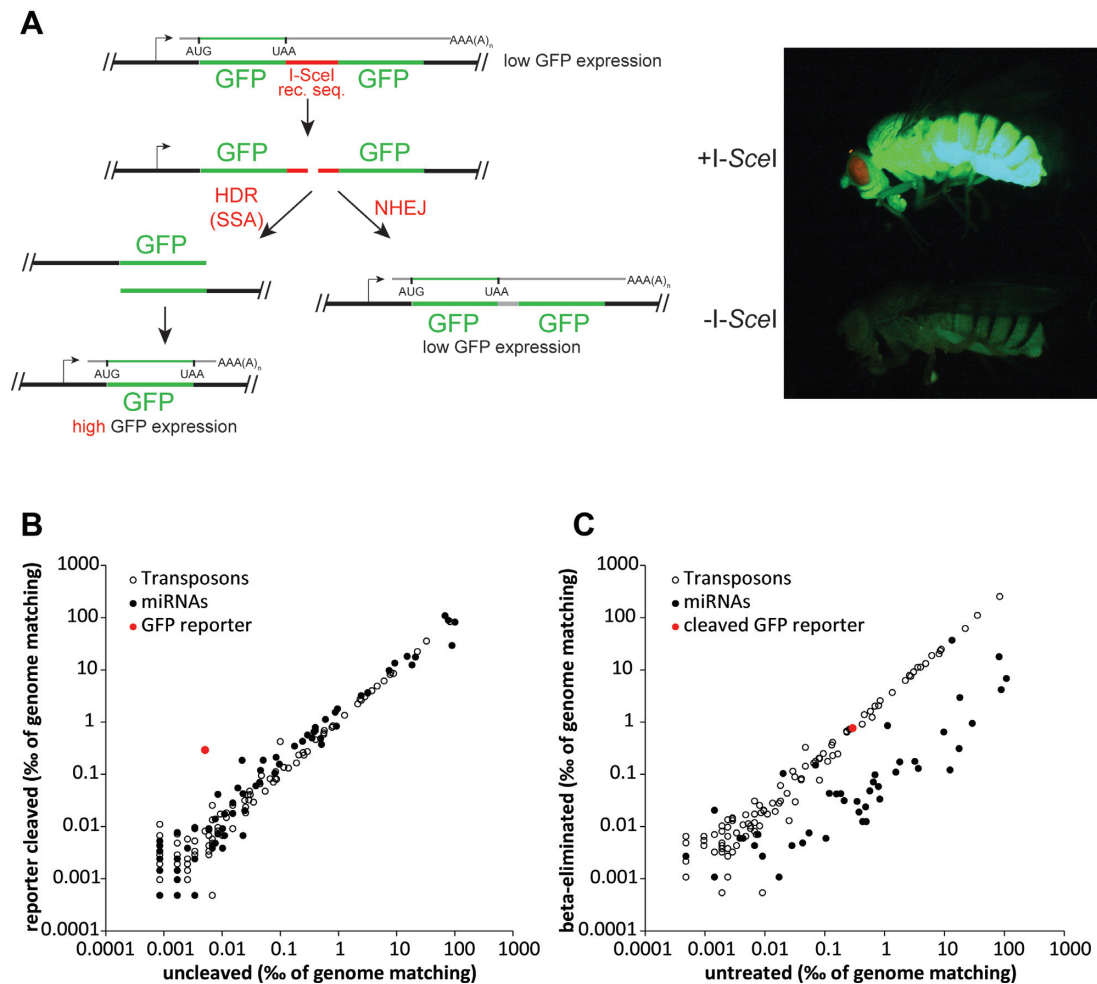
In *Drosophila*, the generation of small RNAs from a DNA end has only been described upon transfection of linearized plasmids. We thus established a reporter system that makes use of the highly specific homing-endonuclease *I-SceI* (17) in flies and S2-cells. Similar to a previously used reporter

system in *Arabidopsis* (2), we separated two stretches of full-length GFP coding sequence by an *I-SceI* recognition sequence. In the original state, only the first GFP open reading frame is translated, while the second GFP sequence is part of the 3'-UTR due to the presence of a stop-codon upstream of the *I-SceI* site. Prior to cleavage and repair, the construct yields only low amounts of GFP (Figure 1A). In *Drosophila*, the nonsense-mediated mRNA decay pathway can be triggered by long 3'-untranslated regions (18) and we suspected that the long 3'-UTR (comprised of a GFP sequence and the SV40 polyadenylation signal) renders the transcript susceptible to degradation via mRNA surveillance pathways (19,20). Consistent with this hypothesis, the uncleaved reporter construct can be de-repressed in S2-cells by knock-down of the nonsense mediated decay (NMD) factor *upfl* (Supplementary Figure S1). Upon introduction of the *I-SceI* nuclease, the construct is cleaved between the two GFP coding sequences. Subsequent repair can either proceed via the NHEJ, resulting in no change of GFP expression, or via the SSA pathway, a form of homology directed repair which in this case removes one GFP coding sequence and thus shortens the 3'-UTR. The resulting substantial increase in GFP fluorescence can be visualized in flies with the help of a fluorescence stereomicroscope (Figure 1A, right) and in cells via flow cytometry (12). We verified the rearrangements in individual offspring animals from a fly cross that combined the double-GFP reporter with the *I-SceI* nuclease. The locus can be PCR-amplified and yields a smaller product if a rearrangement via the SSA pathway has occurred. Uncleaved or faithfully repaired loci can be distinguished from those repaired via via mutagenic NHEJ: *In vitro* digestion of the PCR product with *I-SceI* produces two fragments of equal size if a non-modified locus was amplified, while products of mutagenic NHEJ events are resistant to digestion (Supplementary Figure S2).

### Chromosomally integrated reporters generate Ago2-loaded siRNAs upon induction of a DNA double-strand break

Transfected linearized DNA will generate siRNAs if a transcribed region is interrupted by the cut (5). Does this reflect a response that occurs at a chromosomal DNA break? We established stable S2 cell lines carrying our GFP-*I-SceI* reporter construct and introduced the *I-SceI* nuclease via transfection. Three days later, we isolated small RNAs and subjected them to deep sequencing. Induction of the chromosomally located DNA break via *I-SceI* strongly stimulated the generation of siRNAs (Figure 1B). The pattern of siRNA generation from the chromosomally located break is similar to the one we observed previously upon transient transfection (Supplementary Figure S3). Their abundance was roughly 10-fold lower, presumably due to the reduced copy number of specific DNA breaks in the stable cell line compared with transient plasmid transfection.

Are the break-derived siRNAs incorporated into Ago2 effector complexes? We subjected the RNA to periodate oxidation and beta-elimination prior to sequencing. This treatment renders unmodified RNA 3'-OH ends incompetent for adapter ligation and thus increases the abundance of 2'-*O*-methyl modified small RNAs among the sequencing reads (21). Since *Drosophila* siRNAs are 2'-modified only after



**Figure 1.** siRNAs are generated against the SSA reporter upon introduction of a DSB. (A) Schematic overview over the SSA reporter strategy. Two copies of GFP are separated by a recognition site for the I-SceI nuclease. The original construct results in only little GFP expression, since the reporter transcript is targeted by NMD due to its long 3'-UTR. Upon introduction of a DSB, cells repair the break either by NHEJ or SSA. Only the latter will lead to an increase in GFP expression, which can be observed *in vivo* (compare flies with and without I-SceI nuclease expression). (B) The comparison of small RNA libraries after control treatment or transfection of the I-SceI nuclease shows that introduction of a break in the reporter leads to generation of siRNAs against the break. Generation of microRNAs and siRNAs against transposons is unaffected by the break. (C) Comparison of  $\beta$ -eliminated versus untreated control of small RNA libraries after introduction of the break reveals loading of break-derived siRNAs into Ago2, as is the case for transposon mapping reads. Most miRNAs on the other hand, are loaded into Ago1 and thus susceptible to  $\beta$ -elimination.

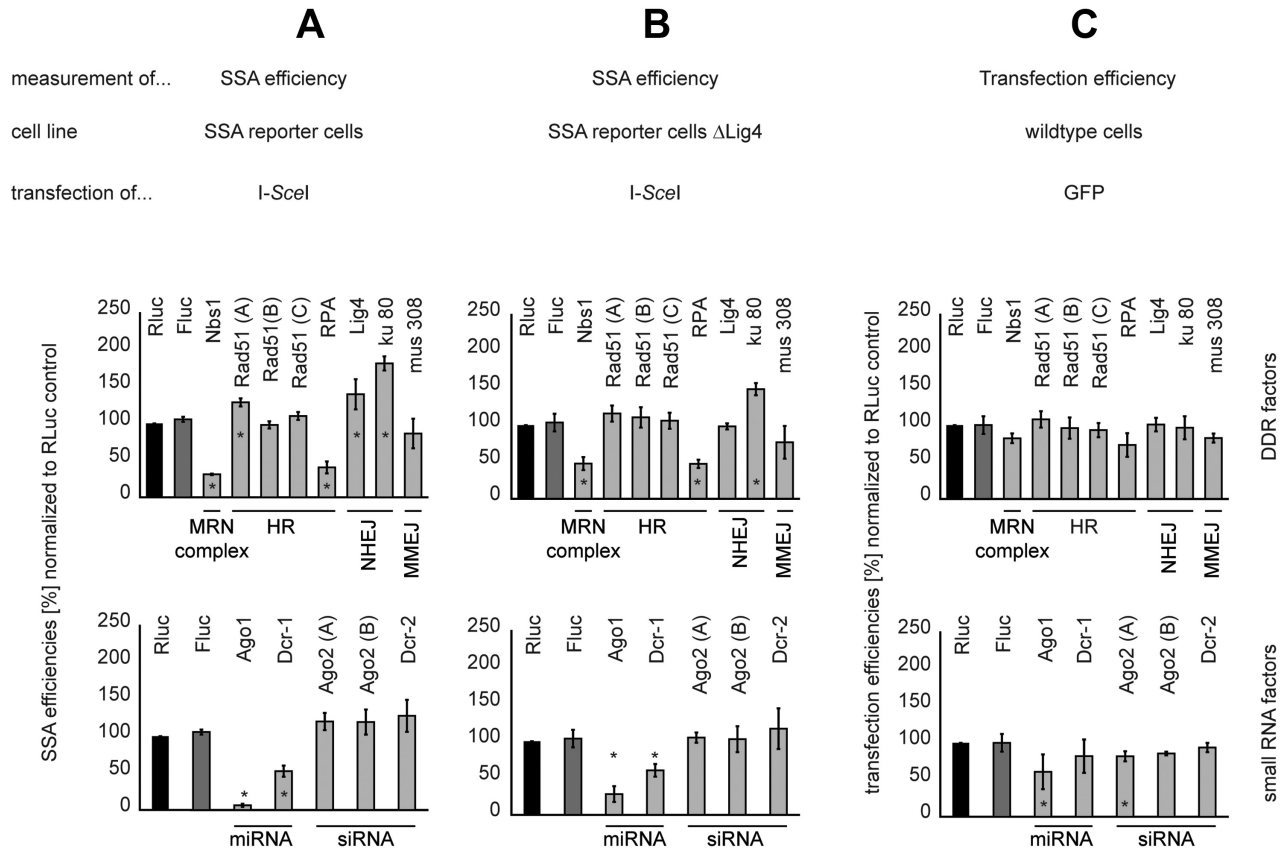
loading into Ago2 (22), enrichment in the sequencing reads obtained from treated RNA demonstrates their incorporation into Ago2 RNAi effector complexes. Break-derived small RNAs were resistant to oxidation and are thus loaded into Ago2 (Figure 1C), consistent with the observation of an Ago2-dependent repressive response at DNA breaks (5). In summary, chromosomally located DNA DSBs in a transcriptionally active region can lead to the production and Ago2-loading of small RNAs with corresponding sequence.

#### The siRNA pathway components have no effect on SSA efficiency

Using RNAi, we depleted our SSA reporter cell lines of factors with a known role in DNA repair, transfected the I-SceI expression plasmid to induce the break and assayed the extent of repair via homologous recombination by flow cytometry. Depletion of the essential HR protein RPA re-

sulted in reduced HR activity as measured by our reporter. Consistent with the notion that SSA does not depend on Rad51 in budding yeast (23), knock-down of Rad51 had no effect on SSA efficiency. On the other hand, depletion of the NHEJ factors Ku80 and Lig4 resulted in increased HR activity, presumably because the competing NHEJ pathway had been attenuated (Figure 2A). Our reporter system thus allows us to detect both increases and decreases in the relative efficiency of HR by SSA.

Having successfully validated our reporter assay, we tested the effect of depleting miRNA pathway components (Ago1, Dcr-1) and siRNA pathway components (Ago2, Dcr-2) (13,15) on DNA repair. Inactivation of miRNA but not siRNA pathway components resulted in an apparent reduction of homologous recombination (Figure 2A and B). However, since induction of the DNA break requires transfection of the I-SceI expression vector, we also measured how knock-down of DNA repair proteins (Nbs1, Rad51,



**Figure 2.** HDR efficiencies of the SSA reporter are unaffected by knock-down of siRNA factors. (A and B) HDR efficiencies were determined by flow cytometry after knock-down of individual factors and subsequent transfection with a plasmid encoding the *I-SceI* nuclease. Knock-down of *Renilla* luciferase (RLuc) served as knock-down control and was used for normalization. Depicted in (A) are HDR efficiencies for the parental SSA reporter cell line, while (B) shows HDR efficiencies for the  $\Delta$ *lig4* SSA reporter cell line. (C) To assess the influence of knockdown of individual factors on the transfection efficiency, we transfected wildtype (S2) cells with a plasmid encoding GFP following the respective knock-down. Five days later, GFP-expressing cells were quantified via flow cytometry. Again, knock-down of RLuc was used as control for normalization. Error bars represent the standard deviation (SD); for Rad51, three independent dsRNA designs were tested, two designs for Ago2. Significant changes compared to the *Firefly* Luciferase (FLuc) are indicated by an asterisk (\*) ( $P < 0.05$ ; two-sided t-Test equal variance,  $n \geq 3$  biological replicates).

RPA, Lig4, Ku80, mus308), miRNA factors (Ago1, Dcr-1) and siRNA factors (Ago2, Dcr-2) affected the transfection (Figure 2C). We found that part of the Ago1 knock-down effect can be explained by a reduced transfection efficiency. Furthermore, as reported by Liu *et al.*, miRNA factor depletion can lead to cell cycle abnormalities in human cells which in turn influence HR repair (8). Indeed, knock-down of the miRNA pathway components Drosha and Ago1, but not the siRNA pathway components Dcr-2 or Ago2, resulted in a pronounced increase of the G1 population in cultured *Drosophila* cells (Supplementary Figure S4). It is thus likely that the apparent reduction in HR repair events was the consequence of changes in the cell cycle distribution, augmented by a lower transfection rate in the case of Ago1. This certainly does not exclude a further contribution of other miRNA-related regulatory events.

The siRNA pathway also processes hairpin-RNAs (24), a class of small RNAs that originate from genomically encoded long RNA hairpins. An abundant small RNA derived from the CG4068 hairpin represses the *mus308* gene, which encodes the *Drosophila* homolog of the mammalian DNA polymerase theta (24–26). This polymerase mediates

an alternative end-joining mechanism that relies on microhomologies (27). It is therefore formally possible that depletion of siRNA factors leads to up-regulation of *mus308*, and that this compensates for changes in HR pathway activity because our SSA reporter is analogously re-arranged via the hyper-activated MMEJ system. We reasoned that upon genetic ablation of *lig4*, *mus308* should always be the predominant end-joining activity. In other words, although the balance between HR and end-joining is overall shifted toward HR in *lig4* deletion cells, the potentially compensating effect of MMEJ after RNAi factor knock-down should be abolished. We used *cas9*-CRISPR to mutagenize the *lig4* gene in our reporter cell line, then re-cloned the cells and analyzed the *lig4* locus by PCR. We recovered one clone that harbored a deletion of  $\sim 0.8$  kB in the vicinity of the *lig4* gene, removing its 5' UTR and the first 10 aminoacids of the N-terminus (Supplementary Figure S5). We then measured the efficiency of SSA after knock-down of various factors and subsequent transfection of *I-SceI* in the  $\Delta$ *lig4* mutant reporter cell line (Figure 2B). Similar to wildtype cells, we observed decreased SSA efficiency after knock-down of RPA, but not after depletion of Rad51. Knock-down of Ku80 re-

sulted in increased SSA efficiency while, as predicted, depletion of *lig4* by RNAi had no effect in the *lig4* mutant cells (Figure 2B). Importantly, also in the *lig4* mutant cell line depletion of RNAi factors had no effect, while knock-down of miRNA factors led to an apparent reduction of SSA efficiencies that may be explained by indirect effects (see above). We thus conclude that homology directed repair via the SSA pathway functions independently of break-derived siRNAs in cultured *Drosophila* cells.

### ***dcr-2* mutant flies have normal levels of homology directed repair**

We repeated our analysis *in vivo* by crossing *dcr-2* mutant alleles to flies that transgenically harbored our SSA reporter construct. We crossed trans-heterozygous *dcr-2<sup>L811fsX</sup>/dcr-2<sup>G31R</sup>* or *dcr-2<sup>L811fsX</sup>/dcr-2<sup>G31R</sup>* females carrying the DSB reporter to *dcr-2<sup>L811fsX</sup>/CyO* or *dcr-2<sup>G31R</sup>/CyO* mutant males carrying a ubiquitously expressed I-*SceI* nuclease transgene. Upon activation of the zygotic genome, the nuclease will thus cleave the GFP reporter and repair by SSA will lead to patches of high GFP fluorescence in the F1 adults (individual patches are not discernible in Figure 1A, right). We quantified the GFP fluorescence in the eye as a proxy for the balance between HR and end joining events in mutant and control animals. Consistent with the cell culture data, there was no difference in the rate of homology-directed repair between maternal and zygotic *dcr-2* mutant animals and heterozygous controls (Figure 3A and B). To obtain an independent measure of GFP expression, we also extracted protein from individual whole flies and quantified the total amount of GFP by Western blotting (Figure 3C). Again, no difference between *dcr-2* mutant and control animals was evident. Taken together, our results obtained *in vivo* with mutant flies do not suggest any requirement of Dcr-2 for SSA-mediated repair.

### **Homologous recombination is unaffected by depletion of siRNA factors**

SSA is a rather specific case of homology-directed repair and may not be fully representative of homologous recombination. In order to test whether homologous recombination repair with a template *in trans* is affected by depletion of siRNA biogenesis factors, we exploited our approach to tag proteins at their genomic locus via the CRISPR/Cas9 system (12). For our assay, we programmed Cas9 to introduce a DSB adjacent to the stop codon of the *act5C* gene. We provided not only the guide RNA, but also a template for HR repair by transfection. To enable readout via flow cytometry, the two homology regions are flanking a GFP coding sequence. Upon integration of our HR donor at the *act5C* gene, a strong fluorescence signal from the fusion protein can be observed in individual cells and quantified by flow cytometry (11). Since there is evidence that Rad51 independent HR repair might be favored for short homology regions (<100 bp) (28) we varied the HR donor length upstream and downstream of the DSB (60/60 bp and 500/420 bp). Additionally, we tested the role of HR donor topology (linear and circular).

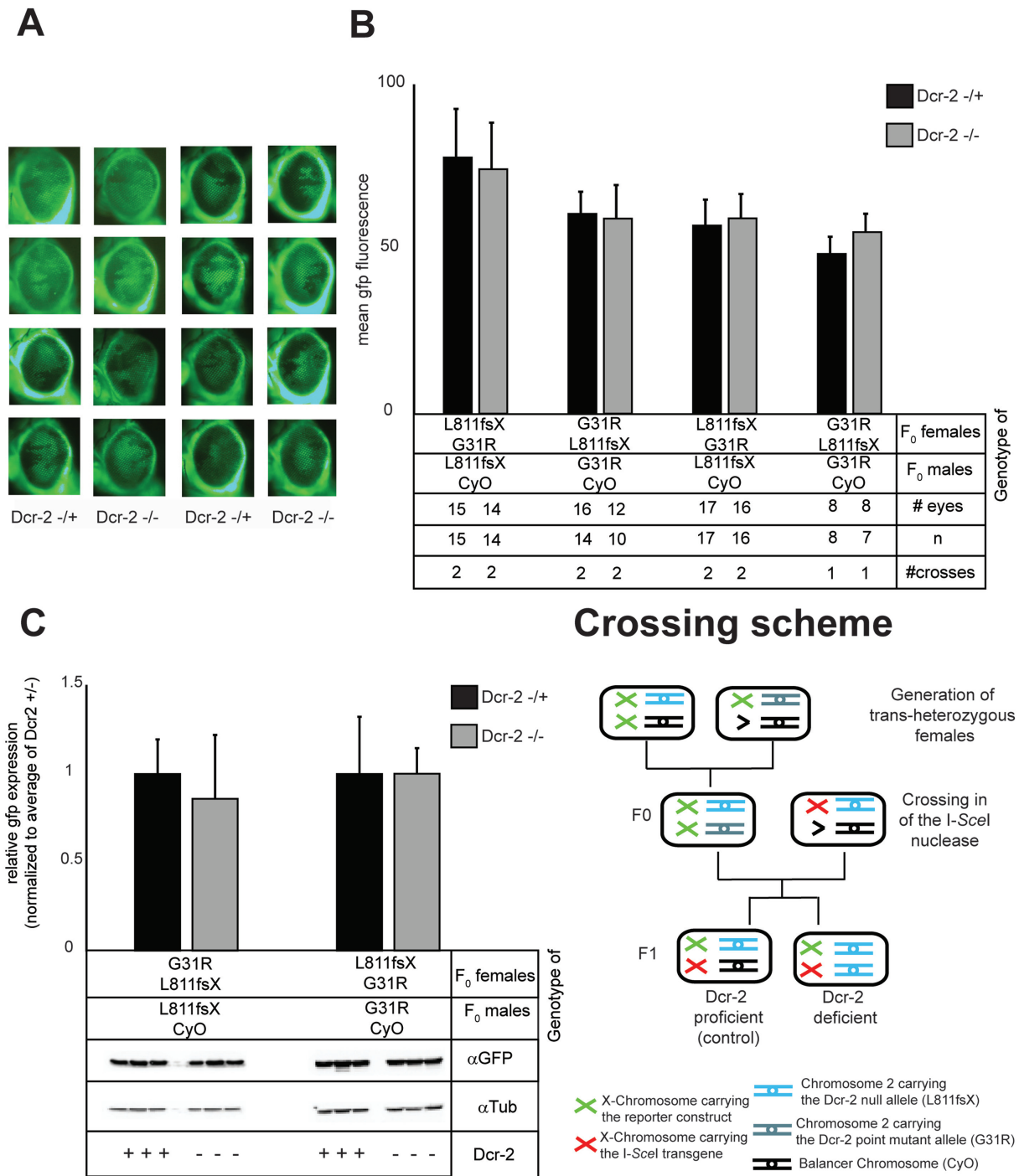
We compared the efficiency of the GFP knock-in after knock-down of the MRN-complex member Nbs1, known

HR factors (RPA, Rad51), NHEJ factors (Lig4, Ku80), miRNA pathway components (Ago1, Dcr-1), as well as siRNA factors (Ago2, Dcr-2) (Figure 4). As before, we observed no decrease in the efficiency of HR upon depletion of siRNA factors, while absence of Nbs1 and RPA clearly reduces HR efficiency. Our results provide insight into the HR donor requirements for engagement of Rad51: Upon knock-down of Rad51 with three independent Rad51 dsRNA designs, we found no strong effect with linear donor molecules bearing either short or long homology arms; the relative HR efficiencies did not fall below  $62.2 \pm 3.4\%$  (short homologies, Figure 4A) and  $64.01 \pm 4.5\%$  (long homologies, Figure 4B) of the control treatment. In contrast, when cells were provided with a circular HR donor with long homology arms for repair, Rad51 depletion had a more pronounced effect and HR efficiencies dropped to  $23.8 \pm 3.1\%$  of the control treatment (Figure 4C). Most importantly, though, we did not observe any reduction in HR with either linear or circular donors upon depletion of the siRNA factors Dcr-2 and Ago2; if anything, the HR efficiency appeared increased after siRNA pathway inhibition (Figure 4, bottom panel). However, this is likely due to technical reasons since detection of Act5C-GFP expression by FACS is facilitated by the absence of repressive siRNAs from the locus (note that the initial recombination frequencies were measured without marker selection).

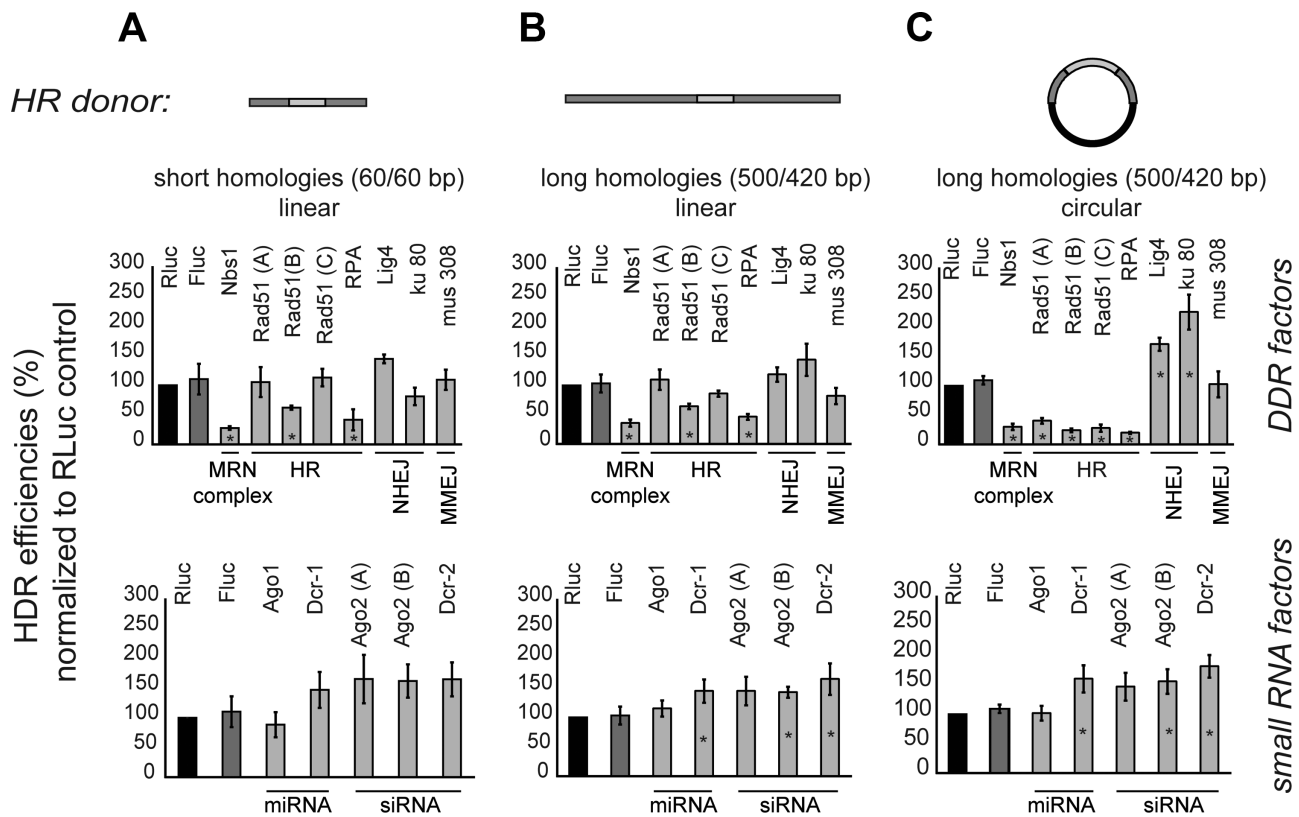
### **Response to DSB stress is independent of Dcr-2**

Given that faithful HR repair both *in cis* and *in trans* is independent of break derived siRNAs, we investigated whether Dcr-2 might be important for a general DSB stress response. To this end, we treated *dcr-2* mutant flies with the topoisomerase I inhibitor camptothecin (CPT). CPT treatment results in the formation of a covalent link between the nicked DNA and topoisomerase I, thus preventing re-ligation. Replication forks that run into this complex collapse, which ultimately leads to the formation of DSBs at numerous sites throughout the genome. This stands in contrast to the single, directed DSBs we introduced for our HR assays. Flies with a mutation in the *brca2* gene, which encodes an important factor for Rad51-dependent homologous recombination, are hypersensitive to camptothecin (29).

We reasoned that in the case of an essential role for Dcr-2 in a DSB stress response, *dcr-2* mutant animals should show impaired development to adult flies in the presence of CPT. Thus, a decrease in the proportion of *dcr-2* mutant flies recovered from a cross of heterozygous parents should be indicative of CPT hypersensitivity (Table 1, see Supplementary Figure S7A for a graphical representation). We did not observe any increased CPT sensitivity with control animals that carried a wildtype chromosome 2 in combination with a *CyO* balancer (Table 1, cross 1). In contrast, treatment with CPT severely reduced hatching of *dcr-2* deficient animals (Table 1, cross 2 and 3). However, we were not able to rescue this phenotype by an ectopically placed and functionally validated *dcr-2* wildtype rescue transgene (6) (Table 1, cross 4). Since both alleles used in our experiments (individually and the trans-heterozygous combination) were derived from the same genetic screen (6), the ob-



**Figure 3.** HDR efficiency of the SSA reporter is unaffected in maternal and zygotic *dcr-2* mutants. The crossing scheme shows the process to generate maternal and zygotic *dcr-2* mutants. First trans-heterozygous females that carry two *dcr-2* mutant alleles are generated to diminish the influence of potential background mutations. Trans-heterozygous females were then used in the consecutive cross with heterozygous males that carry the I-SceI transgene. Progeny (F1) was then subjected to (A and B) fluorescence microscopy and (C) Western Blotting. Heterozygous F1 animals were used as control. (A) Example pictures of fly eyes in adult F1 flies. *dcr-2* proficient flies (Dcr2 +/-) were compared to *dcr-2* deficient flies (Dcr2 -/-). (B) Quantification of HDR efficiency of the SSA reporter was measured via the mean fluorescence within the fly eye using ImageJ. Black bars show the mean GFP fluorescence in *dcr-2* proficient controls, grey bars the fluorescence in *dcr-2* deficient flies. The genotype of F<sub>0</sub> females and males is indicated, as are the number of eyes measured, the number of flies (n) and the number of independent crosses. Error bars show the SD. C) Quantification of Western Blot experiments to assess the relative GFP expression (normalized to tubulin as loading control) within the whole fly. Again, black bars show the GFP protein levels in *dcr-2* proficient animals, while the results for *dcr-2* deficient flies are depicted in grey; error bars represent the SD of three biological replicates. Depicted are also the blots used for quantification. For each lane, whole fly protein extract of a single *dcr-2* proficient (+) or *dcr-2* deficient (-) animal was applied.



**Figure 4.** HDR efficiencies of genome editing are unaffected by knock-down of siRNA factors. Top: schematic representation of the HR donors employed; homology arms are not drawn to scale. S2-cells stably expressing the Cas9 protein were transfected with an sgRNA expression construct and the indicated homology donor to append a GFP moiety to the Act5C gene at its chromosomal locus. Seven days later, cells were analyzed by flow cytometry and the Act5C-GFP positive population was quantified (absolute values were 3–8% for the RLuc control). Knock-down of *Renilla* luciferase (RLuc) served as control for normalization. We tested three independent knock-down constructs for Rad51, two for Ago2. The asterisk (\*) indicates significant changes ( $P < 0.05$ ; two-sided t-Test equal variance,  $n \geq 3$  biological replicates). (A) PCR-based HR donor with 60 nt of homologous sequence at either end; although the MRN complex and RPA appear to be required for homology-directed repair with this donor, the role of Rad51 is limited. Only one knock-down construct resulted in a significant reduction; for the small RNA biogenesis factors no significant change was observed. (B) Extension of the homology arms in the linear configuration (targeting vector pIS1 linearized) results in values largely comparable with the ones observed in (A). For the small RNA factors, a slight apparent increase of HR efficiency is detected; we assume this to be a technical artefact because detection of Act5C-GFP is facilitated in the absence of repressive siRNAs generated at the break. (C) If the HR donor is supplied as a circular targeting vector, the dependency on Rad51 is far more pronounced. In this case, all three knock-down constructs resulted in a highly significant reduction of HR efficiency. For the small RNA biogenesis factors, however, the results are essentially unchanged.

served hypersensitivity is most likely due to a background mutation present in the strain that was mutagenized. We therefore attempted to combine the alleles with independent *dcr-2* mutant chromosomes. The deficiency *Df(2R)ED3385* comprises ~118 kb surrounding the *dcr-2* locus. Trans-heterozygous flies with the *dcr-2<sup>L811fsX</sup>* allele remained hypersensitive to CPT (Table 1, cross 5) but again the rescue with a wildtype *dcr-2* transgene was not successful (Table 1, cross 6). In contrast, a trans-heterozygous combination of the *piggyBAC* transposon insertion *dcr-2<sup>J06544</sup>* with either the deficiency *Df(2R)ED3385* or *dcr-2<sup>L811fsX</sup>* had no increased CPT sensitivity (Table 1, cross 7 and 8). This transposon insertion is located 29 nt downstream of the annotated transcription start site and the allele is dysfunctional in transgenic RNA interference (Supplementary Figure S6). Finally, we found no CPT sensitivity conferred by the catalytically inactive *ago2<sup>V966M</sup>* mutation (30) in trans-heterozygous animals with the *ago2<sup>321</sup>* P-element insertion (31) (Table 1, cross 9). We conclude that an impaired RNAi pathway does not lead to hypersensitivity to the DSB in-

ducing agent camptothecin. The simplest interpretation of our results is that the background mutation responsible for CPT-sensitivity in certain *dcr-2* alleles is either also present on or resides within the region deleted in *Df(2R)ED3385*.

A recent publication described frequent variants of the *cyp6d2* gene that either impair splicing (SD allele) or transcript accumulation (NT allele) and, in both cases, lead to camptothecin hypersensitivity (29). We thus used the published PCR-based assay to test the stocks from our camptothecin experiments. We detected the presence of the SD allele in the *dcr-2<sup>L811fsX</sup>* and *dcr-2<sup>G31R</sup>* strains (including those employed for the transgenic Dcr-2 rescue cross). Furthermore, we found the NT allele in the *Df(2R)ED3385* and *ago2<sup>V966M</sup>* stocks. In contrast, the *dcr-2<sup>J06544</sup>* *piggyBAC* allele and the *ago2<sup>321</sup>* allele did not carry any of the described *cyp6d2* alleles (Supplementary Figure S7B). Thus, all genetic combinations that showed hypersensitivity to camptothecin were also homozygous for *cyp6d2* mutations, while none of the combinations with a functional *cyp6d2* copy was hypersensitive for this drug. Taken together, our anal-



**Table 1.** Camptothecin-challenge of *dcr2* and *ago2* mutant flies (*CyO* and *TM3* are homozygous lethal balancer chromosomes with functional *Dcr-2* or *Ago2* alleles and a visible marker)

cross	genotypes	phenotype	expected ratio	control	DMSO	0.01 mM	0.03 mM	0.1 mM	0.3 mM
1	<i>+/CyO</i>	<i>Cy</i>	2	75	43	112	142	69	84
	<i>x</i>	<i>non-Cy</i>	1	54	38	94	70	38	52
	<i>+/CyO</i>	# crosses		(1)	(2)	(3)	(3)	(2)	(3)
2	<i>dcr2<sup>G31R</sup>/CyO</i>	<i>Cy</i>	2	234	236	105	74	220	131
	<i>x</i>	<i>non-Cy</i>	1	100	141	62	41	21	0
	<i>dcr2<sup>L811fsX</sup>/CyO</i>	# crosses		(3)	(2)	(2)	(2)	(3)*	(3)(*)
3	<i>dcr2<sup>L811fsX</sup>/CyO</i>	<i>Cy</i>	2	155	157	8	100	219	135
	<i>x</i>	<i>non-Cy</i>	1	61	82	2	5*	2	0
	<i>dcr2<sup>L811fsX</sup>/CyO</i>	# crosses		(3)	(2)	(1)	(2)	(3)*	(3)(*)
4	<i>dcr2<sup>L811fsX</sup>/CyO</i>	<i>Cy</i>	1	219	186	183	117	31	20
	<i>x</i>	<i>non-Cy</i>	1	191	163	196	68	1	0
	<i>dcr2<sup>L811fsX</sup>; P{Dcr2}</i>	# crosses		(4)	(3)	(5)	(4)	(1)*	(1)(*)
5	<i>dcr2<sup>L811fsX</sup>/CyO</i>	<i>Cy</i>	2	62	55	45	108	126	23
	<i>x</i>	<i>non-Cy</i>	1	41	36	19	37	4	0
	<i>dcr2<sup>Df(2R)ED3385</sup>/CyO</i>	# crosses		(3)	(3)	(2)	(3)	(3)*	(2)(*)
6	<i>dcr2<sup>Df(2R)ED3385</sup>/CyO</i>	<i>Cy</i>	1	68	49	77	112	95	117
	<i>x</i>	<i>non-Cy</i>	1	81	87	112	114	8	0
	<i>&gt;dcr2<sup>L811fsX</sup>; P{Dcr2}</i>	# crosses		(2)	(3)	(3)	(3)	(3)*	(3)(*)
7	<i>dcr2<sup>06544c</sup>/CyO</i>	<i>Cy</i>	2	93	81	66	18	12	62
	<i>x</i>	<i>non-Cy</i>	1	51	43	37	23	9	25
	<i>dcr2<sup>Df(2R)ED3385</sup>/CyO</i>	# crosses		(2)	(2)	(2)	(2)	(2)	(2)
8	<i>dcr2<sup>06544c</sup>/CyO</i>	<i>Cy</i>	2	175	92	100	230	146	137
	<i>x</i>	<i>non-Cy</i>	1	83	52	69	135	89	66
	<i>dcr2<sup>L811fsX</sup>/CyO</i>	# crosses		(4)	(3)	(3)	(5)	(3)	(4)
9	<i>ago2<sup>321</sup> / TM3, Sb</i>	<i>Sb</i>	1	51	85	49	72	60	58
	<i>x</i>	<i>non-Sb</i>	1	64	89	56	111	91	52
	<i>ago2<sup>V966M</sup></i>	# crosses		(2)	(2)	(2)	(3)	(3)	(3)

\*distribution different from expected,  $P < 10^{-5}$ ,  $\chi^2$  test.

(\*)no exact  $\chi^2$  test calculation possible because of 0 observed events in one category.

ysis demonstrates that the camptothecin hypersensitivity in certain RNAi mutant stocks is caused by *cyp6d2* background mutations.

## DISCUSSION

In this manuscript we examined the requirement of *dcr-2* for efficient DNA double-strand break (DSB) repair in *Drosophila melanogaster*. We present a GFP-based system that reports on the balance of homology-directed repair versus non-homologous end joining upon induction of a single break with the *I-SceI* nuclease. The reporter is established both in a stable, clonally derived S2-cell line and in transgenic flies. With this reporter, we demonstrate that a break located in the chromosome induces an siRNA response that is very similar to the one we had observed previously with transfected linearized plasmids (5). This is in accordance with reports in *Neurospora crassa*, *Arabidopsis thaliana* and human cell culture, demonstrating the conservation of the siRNA response to a DSB within a transcribed region. Cells of *Neurospora* are indeed hypersensitive to DNA-damaging conditions if important RNAi-factors are mutated (9). Furthermore, there is a genetic in-

terdependence between homologous recombination and the generation of the damaged-induced qiRNAs from repetitive sequences in *Neurospora* (10).

In *Arabidopsis* and human cells, a link between DSB induced small RNA biogenesis and efficient DSB repair was also proposed (2) and a physical interaction between mammalian Rad51 and Ago2 was recently described (3). A potential caveat of work with *A. thaliana* and mammalian cells is that the small RNA biogenesis pathways are intertwined. In *Arabidopsis*, several classes of small RNAs with distinct functions have been described. Although they may be preferentially produced by one of the four Dicer-like proteins and loaded into a specific Argonaute effector, none of the Dicer-like proteins produces exclusively a single class of siRNAs (32); genetic ablation may therefore affect more than one pathway. In mammalian cells, a single Dicer enzyme produces both siRNAs and miRNAs. Consequently, Dicer mutant mice are not viable. Analogously, although there are four distinct Argonaute proteins in mammals, only Ago2 is catalytically active and the major acceptor of both, miRNAs and siRNAs. Depletion of Ago2 and Drosha, as reported by Wei *et al.* (2), could thus indirectly affect how DNA damage is dealt with since miRNAs target DNA re-

pair factors and regulate cell cycle progression. Indeed, a recent publication demonstrated that overexpression of the miRNA *let-7* or related miRNAs rescues the observed HR deficiency in Dicer knock-down cells (8).

We have previously demonstrated that the *Drosophila* DNA DSB induced siRNAs depend on Dcr-2 and Ago2 for their function, while depletion of the miRNA factors Droscha and Dcr-1 had no detectable influence on their generation (5). This was now confirmed by the demonstration that the break-derived siRNAs are fully resistant to periodate oxidation and beta-elimination (Figure 1C), indicating that they all carry the 2'-O-methyl modification at their 3'-end that is characteristic of Ago2-loaded small RNAs. In *Drosophila*, the biogenesis of most miRNAs can be separated from siRNAs because Dcr-1 is dedicated to the processing of miRNAs, while Dcr-2 produces siRNAs from long double-stranded RNA (6,15,33). Similarly, the Ago1 protein is the essential miRNA acceptor while Ago2 is, like Dcr-2, non-essential. Nonetheless, male flies with a mutation in *ago2* or *dcr-2* show impaired fertility due to reduced expression of hairpin RNAs (34). In addition, zygotic defects in chromatin structure and mitosis were reported for *ago2* mutant embryos (35). Thus, *Drosophila* offers the possibility to study the effect of siRNA ablation with most likely marginal side-effects on the miRNA pathway. We therefore developed a SSA reporter analogous to the ones employed in *Arabidopsis* to probe HR efficiency between two repeats of GFP coding sequence. Both in *dcr-2* mutant flies and in cells depleted for siRNA factors, we found that SSA repair was unaffected. Knock-down of miRNA factors, however, led to an apparent reduction of SSA efficiencies; a likely reason for this is a shift in cell cycle distribution and proliferation as well as a decrease in transfection efficiency (see Figure 2 and Supplementary Figure S5).

SSA is a special subtype of homology directed repair; we also tested whether break-induced siRNAs influence the efficiency of repair when homologous recombination occurs with a donor *in trans*. Using a GFP knock-in approach as reporter for HR repair, we were able to confirm the observation that efficient HR repair is independent of an siRNA response at the DSB. We noticed that the requirement for Rad51 was particularly strong in case of circular HR donors, while the siRNA pathway components showed no change for any of the HR donors employed. Finally, a general DSB stress response to camptothecin treatment, which results in the creation of many DSBs scattered throughout the genome, appeared unaffected by mutation of *dcr-2 in vivo*. This experiment was complicated by a background mutation of the *cyp6d2* gene in certain strains that renders the flies hypersensitive to CPT in a *dcr-2* independent manner as demonstrated with an ectopic rescue transgene (Table 1). We confirmed that the sensitivity to CTP was not caused by the *dcr-2* mutations with the help of a number of different *dcr-2* alleles in trans-heterozygous combinations and PCR-based genotyping for known *cyp6d2* alleles (Supplementary Figure S7). In addition, trans-heterozygous *ago2* mutant flies also showed no CPT hypersensitivity. Taken together, we conclude that in *Drosophila melanogaster* the outcome of DNA DSB repair does not depend on break-derived siRNAs. It is a formal possibility that alternative backup mechanisms take over if siRNAs cannot be gen-

erated. This might result in changes that can only be observed during a time-window that is below the resolution of our assays (readout occurs days after induction of the DNA DSB). Nonetheless, any putative role of siRNAs should be of accessory nature because they are only generated when the break occurs within a transcribed region (5), yet chromosomes must also be repaired when they break in between genes. This is corroborated by the fact that *dcr-2* and *ago2* are non-essential in flies. In contrast, mutant alleles of core DSB repair factors are generally either lethal or sterile due to problems with meiotic crossover resolution.

Studies with Dicer knock-out mice also allow some conclusions on other DNA repair pathways: During vertebrate B-cell development, specific DNA DSBs are created by the Rag1/2 proteins to initiate rearrangement of a recombined V(D)J locus; these somewhat atypical DNA DSBs are exclusively repaired by NHEJ. Conditional ablation of murine *dicer* in developing B-cells did not affect repair pathway choice, indicating that the NHEJ pathway is unperturbed in the absence of damage-induced siRNAs. There were, however, indirect effects because miRNAs no longer repressed pro-apoptotic genes and altered transcriptional control of the enzyme terminal deoxynucleotidyl transferase (36). Similar processes may take place in flies, where the MMEJ-factor DNA polymerase theta (*mus308*) may be repressed by a hairpin-derived endogenous siRNA in a *dcr-2* dependent manner (24,26). We have not examined in detail whether this changes the nature of the end-joining products in the absence of *dcr-2* because the focus of our manuscript is on the role of the siRNAs generated at the site of the DNA DSB. We do note, however, that depletion of *lig4* has a stronger influence on the ratio of homology-directed versus end-joining repair than depletion of *mus308* (e.g. Figure 4).

The findings presented in our current study, as well as the previous observation that generation of DSB induced siRNAs depends on transcription, are in line with a role of break-derived siRNAs in RNA quality control rather than DNA repair. Furthermore, the presence of break-derived siRNAs could slow down the kinetics of re-expression after the break has been mended. It is also currently unclear whether *in vivo* a DNA DSB within a transcriptional unit will lead to run-off or rather stalling of RNA polymerase II. In the latter case, the generation of break-derived siRNAs bears at least conceptual similarity to the recently described identification of transposon transcripts through stalled spliceosomes in *Cryptococcus neoformans* (37). Since *Neurospora* qiRNAs are primarily directed against repetitive DNA and thus are potential players in transposon defense, they might also function in a self versus non-self recognition system. Further studies will reveal the precise role of break-derived siRNAs in mRNA quality control as well as the mechanism of dsRNA synthesis at the break.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

## ACKNOWLEDGEMENT

The anti-tubulin antibody E7 was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD

of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242, USA.

## FUNDING

Deutsche Forschungsgemeinschaft (DFG) [SFB646]. Funding for open access charge: Deutsche Forschungsgemeinschaft [SFB 646]; Ludwig-Maximilians-Universität München.

*Conflict of interest statement.* None declared.

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