Supplementary figures

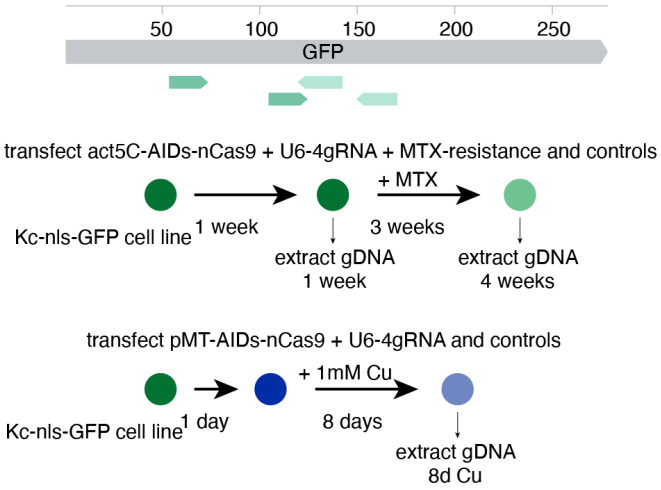

Figure S1. Experimental scheme for cell culture experiments. (A) Binding location of the 4 gRNAs targeting GFP. **(B)** Experimental protocol to induce and assay mutagenesis in cell culture. Timepoints of genomic DNA collection of Kc-NLS-GFP cells after transfection of gRNAs, AID-nCas9 and MTX resistance. Genomic DNA was collected after 1 and 4 weeks for *act5C* driven constructs, and after 9 days in the case of *pMT*. GFP was then amplified and sequenced using Nanopore sequencing.

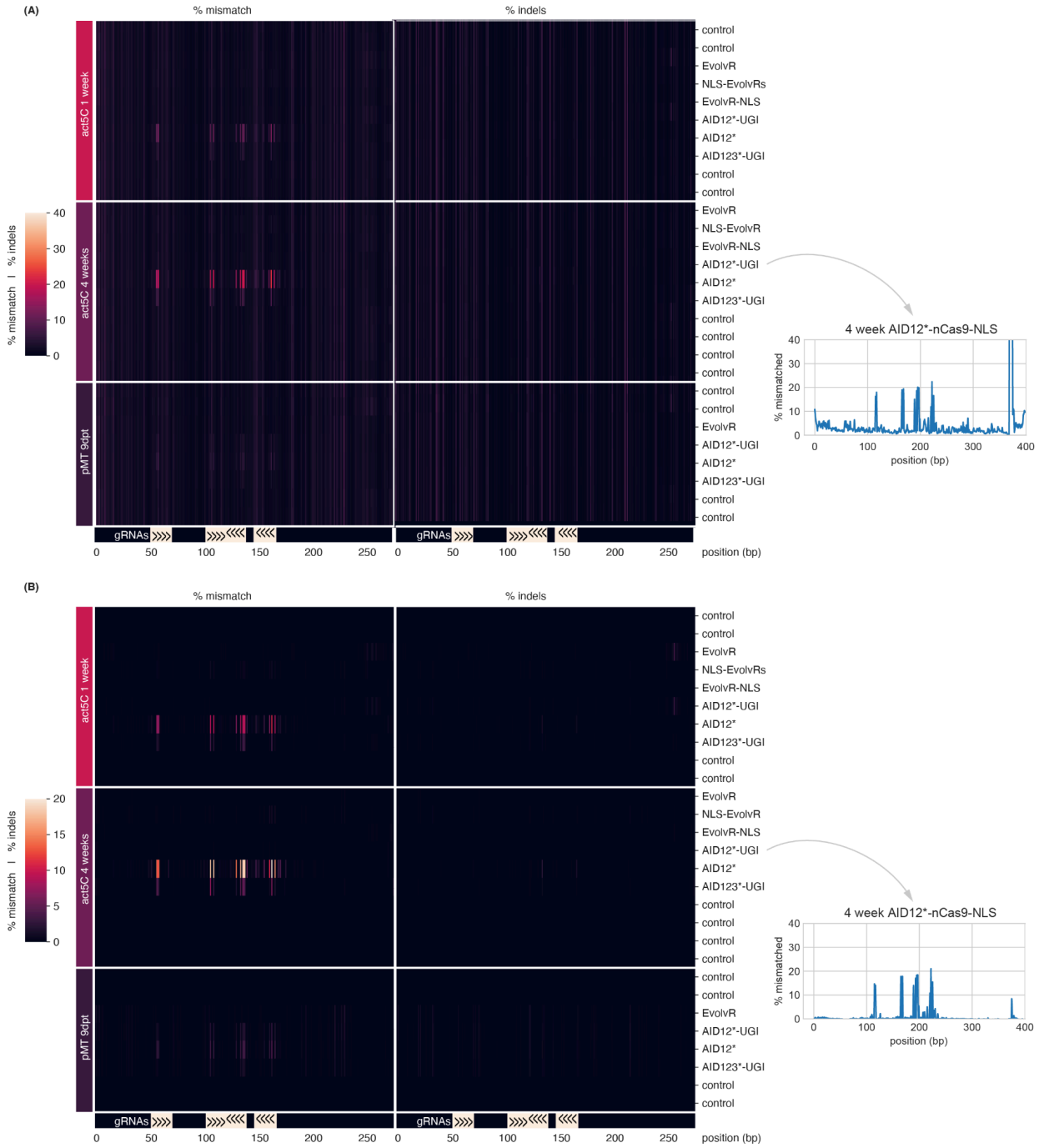

Figure S2. Nanopore sequencing results in cell culture. (A-B) Heatmap showing percentage of mismatch and indels to the GFP sequence before (A) and after (B) subtracting background mutations detected in controls (see methods) in the different timepoints and mutagenic enzyme conditions. AID samples exhibit a high percentage of mismatches aligning with the location of the gRNAs. Right plots show example profiles before and after background subtraction.

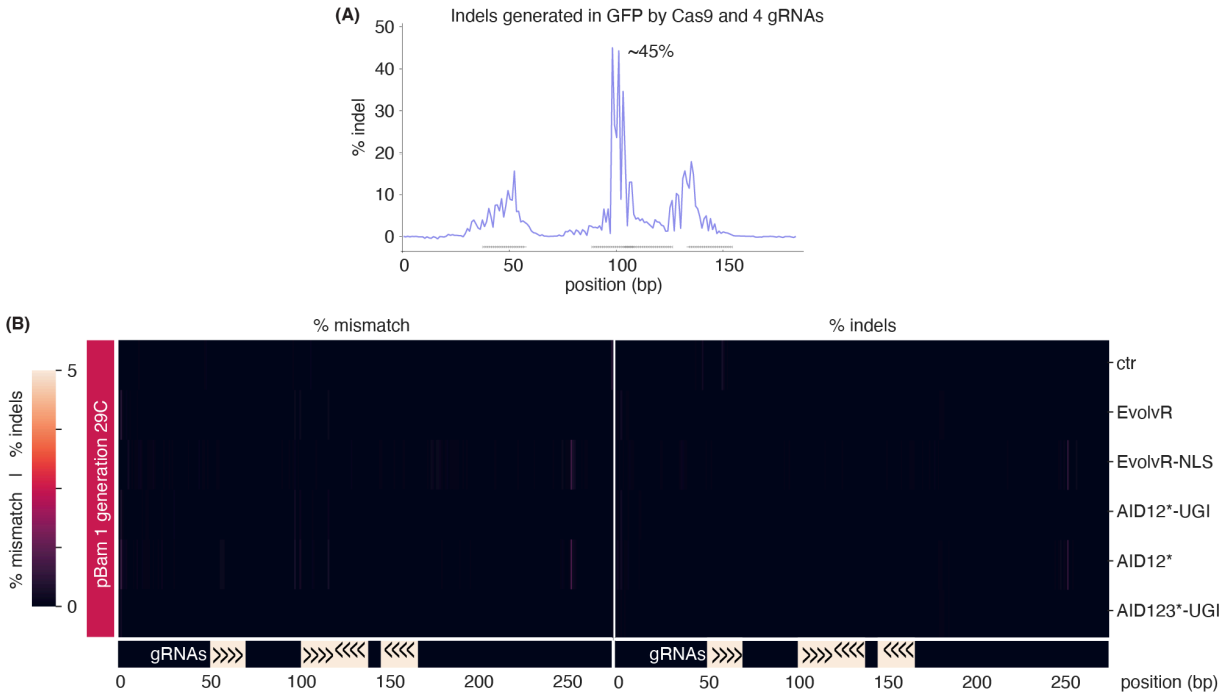

Figure S3. Testing gRNAs using catalytically active Cas9 and mutations in embryos at 29C (A) Catalytically active Cas9 introduces indels at target sites with variable efficiencies, with a maximum efficiency of approximately 45%. Since GFP could have been derived from males or females but *vasa*-Cas9 was only expressed in females, the actual efficiency would be approximately 90%. **(B)** Heatmap showing percentage of mismatch and indels to the GFP sequence after subtracting background mutations detected in controls in embryos where the indicated enzymes could have generated mutations for one generation at 29C. No mismatches or indels were detected.

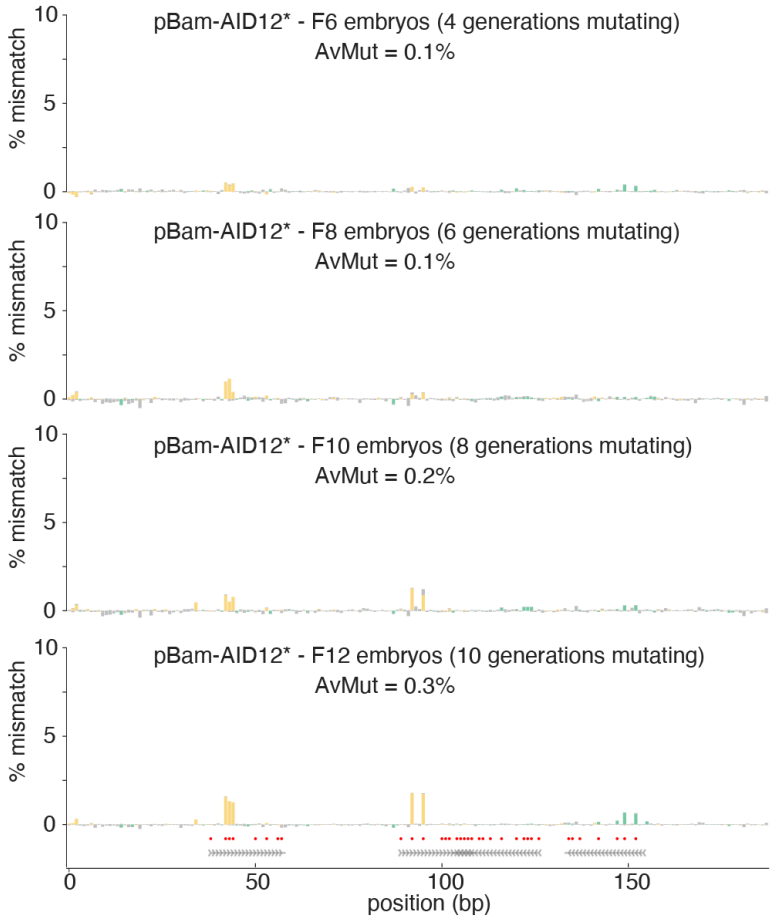

Figure S4. Mutagenesis in flies over multiple generations. Mismatches to GFP classified by base substitution in embryos from stable stocks expression pBam-AID12* and 4 gRNAs targeting GFP collected after increasing numbers of generations over which mutagenesis was carried out.

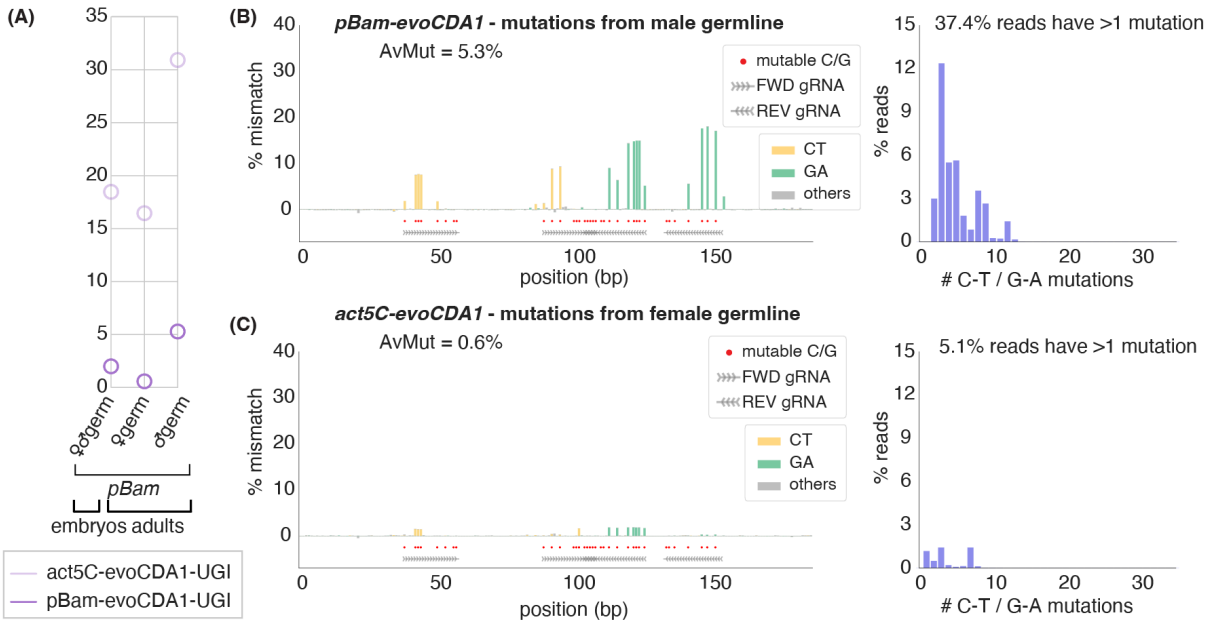

Figure S5. Mutagenesis by lamprey AID^{evoCDA1} expressed from a *bam* promoter. (A) Comparison of average mutation rates obtained by AID^{evoCDA1} expressed from *act5C* or *bam* promoters, in embryos and adults mutated from only the female or male germline. Mutations are significantly lower with *pBam* than *act5C.* **(B)** Mismatches in GFP classified by base substitution in adults were pBam-evoCDA1-nCas9 introduced mutations with the 4 gRNAs from the male (top) or female (bottom) germline (left), and distribution of the observed number of mutations per read after subtracting the number of mismatches observed in control samples (right). Red dots mark all "mutable bases" (Cs on FWD gRNAs and Gs on REV gRNAs).

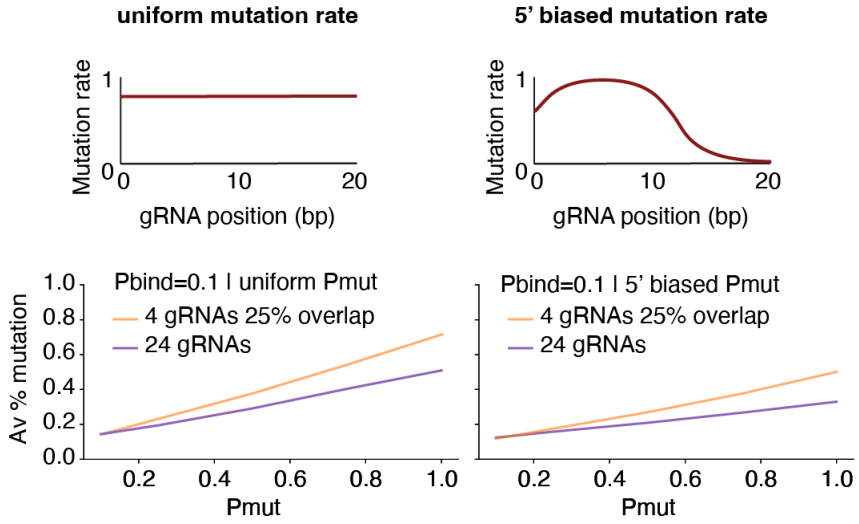

Figure S6. Simulating the effects of uniform and 5' biased mutation rates. Average fraction of mutations at the end of the simulation (generation 100) when comparing 4 and 24 gRNAs targeting GFP, for a range of p_{bound} (different colors) and p_{mut} (x-axis) when mutation profiles were uniform along the gRNA (left) and when a 5' bias was incorporated (right).

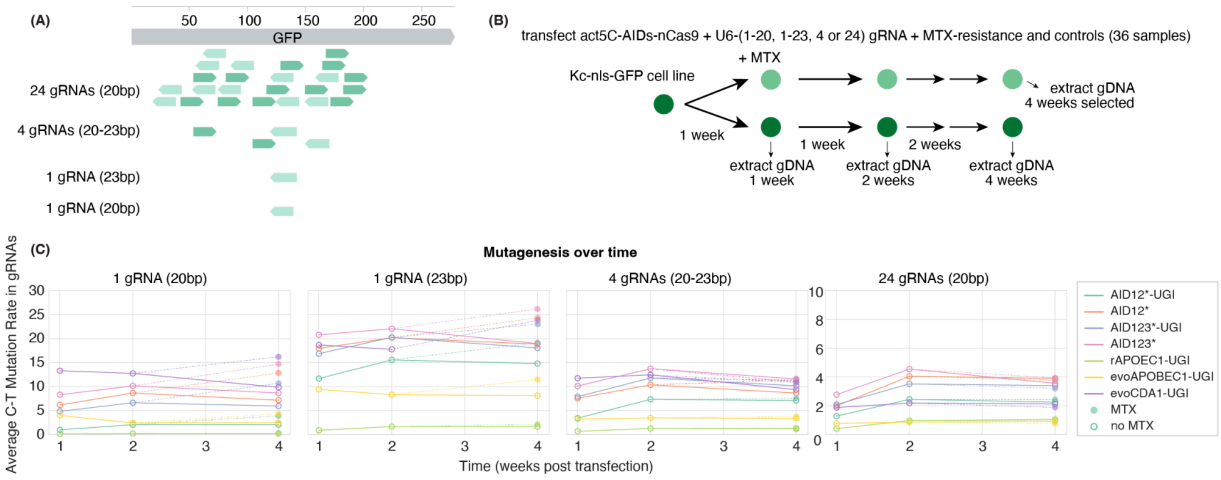

Figure S7. Overview of the experiment in cell culture testing different number and length of gRNA. (A) Scheme of the different gRNA array constructs used to target GFP. **(B)** Scheme of the timepoints where genomic DNA was collected from Kc-GFP lines after transfection of mutagenic enzymes and gRNAs. We attempted to make stable cell lines by co-transfecting with an MTX resistance plasmid and adding MTX (see methods). **(C)** Average mutation rates obtained with each gRNA plasmid (left to right), mutagenic enzyme (lines of different colors) and timepoints (x-axis).

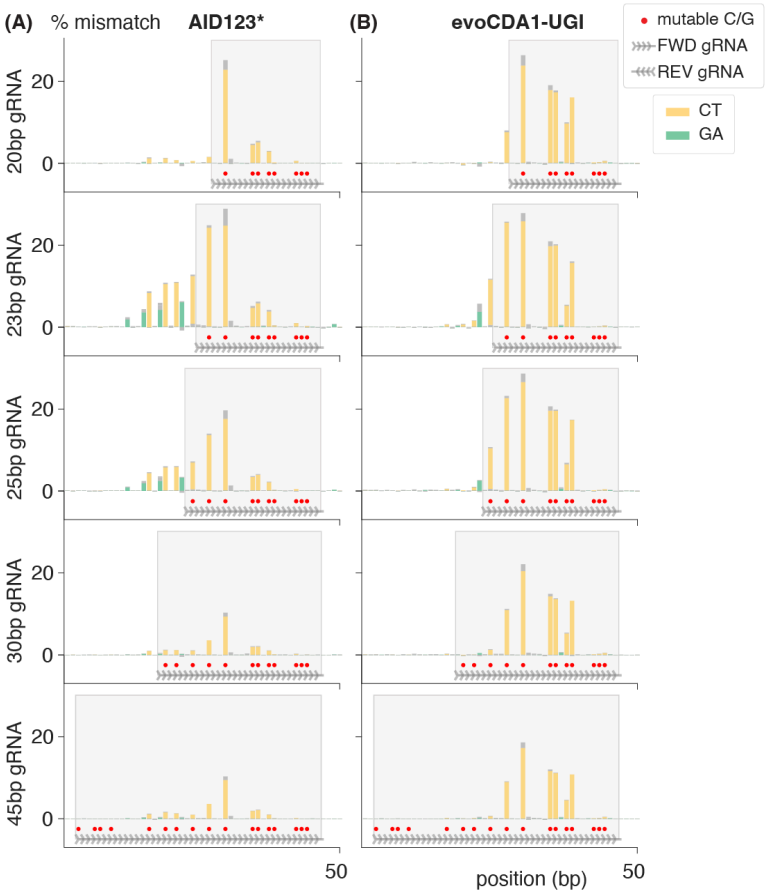

Figure S8. Increasing gRNA length beyond 23bp does not further increase efficiency. (A) Mismatches produced in GFP classified by base substitution in Kc cells transfected with act5C-AID123*-nCas9 and one gRNA of 20, 23, 25, 30 or 45bp in length, 1 week post-transfection. **(B)** Mismatches to GFP classified by base substitution in Kc cells transfected with act5C-AID^{evoCDA1}-nCas9 and one gRNA of 20, 23, 25, 30 or 45 bp in length, 1 week post-transfection.