Mutagenesis and homologous recombination in Drosophila cell lines using CRISPR/Cas9

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Summary

We have applied the CRISPR/Cas9 system to Drosophila S2 cells to generate targeted genetic mutations in more than 85% of alleles. By targeting a constitutive exon of the AGO1 gene, we demonstrate homozygous mutation in up to 82% of cells, thereby allowing the study of genetic knockouts in a Drosophila cell line for the first time. We have shown that homologous gene targeting is possible at 1–4% efficiency using this system, allowing for the construction of defined insertions and deletions. We demonstrate that a 1 kb homology arm length is optimal for integration by homologous gene targeting, and demonstrate its efficacy by tagging the endogenous AGO1 protein. This technology enables controlled genetic manipulation in Drosophila cell lines, and its simplicity offers the opportunity to study cellular phenotypes genome-wide.

Introduction

Genome engineering technologies permit precise alterations of eukaryotic genomes thereby enabling more directed and more nuanced studies of gene function. The ability to perform such manipulations in essentially any organism has been driven by the use of nucleases that can be targeted to specific sites within the genome in a predictable manner (reviewed by Gaj et al., 2013). These can create double strand breaks (DSB) leading to enhanced rates of DNA repair at the targeted site by either non-homologous end joining (NHEJ) or homologous recombination (HR) (Bibikova et al., 2002). Both mechanisms can be exploited to study gene function. The NHEJ repair mechanism occasionally results in the insertion or deletion of a few bases at the DSB site, which can shift reading frame in proteins or remove functionality from transcription factor binding or splice sites. If supplied with exogenous DNA repair templates, HR can be used to engineer directed changes at defined loci (Beumer et al., 2006; Beumer et al., 2008; Bibikova et al., 2003). The ability to design site-specific nucleases allows the generation of targeted mutations and homologous integrations in systems that have hitherto remained refractory to such manipulations.

The type II CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated) system of viral defence in bacteria (Barrangou et al., 2007; Ishino et al., 1987) has recently been adapted for genome engineering in many organisms including zebrafish (Hwang et al., 2013; Xiao et al., 2013), mouse (Wang et al., 2013; Yang et al., 2013) and Drosophila (Bassett et al., 2013; Gratz et al., 2013; Kondo and Ueda, 2013; Ren et al., 2013; Sebo et al., 2013; Yu et al., 2013).

The Cas9 endonuclease from Streptococcus pyogenes can be targeted using a short synthetic guide RNA (sgRNA) to generate a double strand break at a specific site in the genome (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013b). This sgRNA contains a 20 nucleotide target sequence that determines specificity through complementary base pairing with the DNA. The Cas9 protein additionally requires a protospacer adjacent motif (PAM) of NGG to occur within the DNA adjacent to the target sequence to achieve efficient endonucleolytic cleavage (Fig. 1A). This system has only a short specificity determinant and relatively relaxed targeting rules (Cradick et al., 2013; Fu et al., 2013), making its application to large genomes more difficult due to off target mutagenesis (Cho et al., 2013; Hsu et al., 2013; Ran et al., 2013). This hasprompted development of the “double nick” technique that requires the coordinated activity of a pair of mutated Cas9 proteins targeted to neighbouring sequences, to improve sequence specificity (Mali et al., 2013a; Ran et al., 2013). Whilst this could be applied to smaller genomes such as Drosophila, it is possible that even a single sgRNA may be sufficient so long as sgRNA sequences are carefully chosen to minimise off target effects (Bassett et al., 2013; Gratz et al., 2013; Ren et al., 2013), although more rigorous studies will be necessary to establish the extent and nature of off targeting in Drosophila cells.

The CRISPR/Cas9 system has recently been adapted by us and others to engineer short and long deletions in chosen Drosophila genes (Bassett et al., 2013; Gratz et al., 2013; Kondo and Ueda, 2013; Ren et al., 2013; Sebo et al., 2013; Yu et al., 2013). Here we describe its application to Drosophila cell culture. This now allows analysis of cellular phenotypes resulting from the targeted mutation of a gene that may be difficult or impossible to perform...
in the context of a whole organism. This may be due to the complexities of dealing with a mixture of multiple cell types, or embryonic lethality of certain mutations preventing analysis at an appropriate stage. The simplicity, speed and efficiency of the generation of genetic mutations also allow screening for candidate genes involved in particular cellular processes. This system provides a powerful alternative to currently available RNAi screens, which are limited to providing only partial knockdown of function at a post-transcriptional stage.

Results
A gene deletion system for Drosophila cells
We designed an expression vector similar to those previously used in mammalian cells (Cong et al., 2013) but using the Drosophila U6 promoter (Wakiyama et al., 2005) to drive pol III dependent transcription of a chimeric synthetic guide RNA (sgRNA) and the constitutive actin 5c promoter to drive Cas9 mRNA expression (Fig. 1B). Due to the relatively low efficiency of transfection of Drosophila cell lines we also included a puromycin N-acetyltransferase gene downstream of the Cas9 gene, separated from it by a viral derived 2A ribosome skipping site (González et al., 2011). This enables selection for Cas9 expression with puromycin, since the two proteins are produced as part of the same bicistronic transcript derived from the single actin 5c promoter.

Two Bsp QI restriction enzyme sites were introduced within the sgRNA to enable cloning of a pair of annealed 23–24 nt oligonucleotides that generate the target sequence of the sgRNA. Since this enzyme cuts outside of its recognition site, it removes the recognition site during cloning and thus avoids the insertion of any additional sequence. Oligonucleotides are designed by taking the 20 nt target sequence upstream of the PAM (NGG), and adding complementary overhangs to enable cloning into the Bsp QI site (Fig. 1C). The U6 promoter requires the first base of
the transcript to be a guanine, so this either constitutes the first base of the target sequence, or can be appended to it. Consistent with the results of others, we have observed that this only marginally affects cleavage efficiency by the sgRNA (Cong et al., 2013), and greatly increases the choice of target sites within the genome, which are therefore restricted only by the requirement of the NGG protospacer adjacent motif (PAM) sequence (Fig. 1A).

Highly efficient mutagenesis at the yellow locus

We tested our system by using a sgRNA targeted against the yellow locus, which is known to exhibit high cleavage efficiency in flies (Fig. 2A) (Bassett et al., 2013), and detected the insertion and deletion (indel) mutations generated through imperfect non-homologous end joining (NHEJ) by high resolution melt analysis (HRMA, Fig. 2B,D). As increasing amounts of expression vector were transfected, the numbers of generated indel mutations increased (Fig. 2B). Sequencing of generated indel mutations in PCR products spanning the cleavage site showed that transient expression generated mutations in approximately 11% of alleles at 3 d post transfection (Fig. 2C). Upon selection of these cells for a further 7 d in puromycin to remove untransfected cells and to force integration of the vector into the genome, we observed a large increase in the efficiency of mutagenesis (Fig. 2D). Sequencing across the cleavage site demonstrated mutational events in 88% of alleles (Fig. 2E). Mutagenesis was not observed when empty vector lacking the sgRNA targeting sequence was used (Fig. 2B,D). Recent observations of off target cleavage by the CRISPR/Cas9 system (Cho et al., 2013; Cradick et al., 2013; Fu et al., 2013; Hsu et al., 2013; Ran et al., 2013) prompted us to test for mutagenesis at the site with the closest match to the y1 sgRNA (CG14073). We also observed no detectable off-target

Fig. 2. Mutagenesis of the yellow gene is highly efficient. (A) Schematic of the yellow gene showing position of sgRNA target site. Exons are indicated as black boxes, transcriptional start site by an arrow and the y1 sgRNA target site by a black triangle. (B) Indel detection by high resolution melt analysis (HRMA) after transient transfection. Cells were transfected with empty vector lacking sgRNA (EV, grey lines) or with different amounts of vector expressing the y1 sgRNA (2, 1, 0.5 ng, blue lines). DNA was analysed by HRMA 3 days post-transfection, and indicated as melting curves (upper panel) or change relative to control (lower panel). (C) Sequencing of indel mutations after transient transfection. PCR products spanning the cleavage site (black triangle) were cloned and sequenced from cells transfected with 2 µg y1 sgRNA vector, and showed deletions in 2 of 18 clones sequenced at the expected site. Target site is highlighted in orange, and PAM in red. (D) As panel B for cells selected for a further 7 days in puromycin. (E) As panel C for cells selected for a further 7 days in puromycin. (F) Lack of off target mutagenesis at the CG14073 gene. Base pairing interactions between the y1 sgRNA and the site in CG14073 are indicated (target site indicated in orange, PAM in red, cleavage site as a black triangle). HRMA analysis at the closest off-target site in the Drosophila genome indicates a lack of detectable indel mutants even after 7 days selection in puromycin (as panels D,E). Primer sequences are indicated in supplementary material Table S1.
mutagenesis by HRMA even after 7 d selection in puromycin (Fig. 2F), although we cannot exclude the possibility that this may happen at other sites.

Consequently, puromycin selection for different lengths of time can be used to regulate the proportion of mutant alleles within the population of cells. We note that selection may not be desirable in all instances, because it will result in integration of the vector into the genome. Nevertheless, once indels have been generated by this technique, the target site will be removed and the sgRNA will not be able to target Cas9 to this site, so the integrated vector should be unable to cause further mutational events.

Homologous integration at the yellow locus
We next investigated whether homologous integration is possible in S2 cells, as has been demonstrated for human and mouse cell lines (Chen et al., 2011; Cong et al., 2013; Wang et al., 2013). Previous studies have suggested that both short single stranded (ss) DNA oligonucleotides and longer double stranded (ds) DNA donors can be used as templates for homologous recombination in Drosophila and other organisms (Bedell et al., 2012; Beumer et al., 2013; Chen et al., 2011; Cong et al., 2013; Wang et al., 2013). We therefore cotransfected DNA donors with homology either side of the y1 sgRNA cleavage site with the y1 sgRNA/Cas9 expression vector, and assessed integration efficiency by PCR (Fig. 3). We used dsDNA donor plasmids with approximately 200, 500, 1000 or 2000 bp homology on either side of the cut site to insert a 1.8 kb DNA fragment, or a short 100 nt ssDNA oligonucleotide to insert a 23 nt sequence into the genome (Fig. 3A). Transient expression gave low but detectable integration efficiencies of around 0.3% for the longest homology arm, but after selection in puromycin to enrich for transfected cells this was increased to around 2% of alleles. As homology arm length was decreased, integration efficiency also decreased, and the oligonucleotides were integrated at a low efficiency of approximately 0.15% (Fig. 3B). The optimal length for integration efficiency and ease of detection was a dsDNA donor with 1 kb homology either side of the inserted sequence, consistent with previous results when the DSB was induced with a zinc finger nuclease (Beumer et al., 2013). Since it is possible that DNA sequences may be incorporated by direct ligation at the site of the break, we sequenced PCR products spanning the homology arms. This showed that correct homologous integration had occurred in all cases, including with the short ssDNA oligonucleotides.

Mutations of the AGO1 gene
A useful application of this technique would be to generate genetic mutations in Drosophila cell lines, which hitherto has proved impossible. We attempted to construct null mutations of the AGO1 gene using a sgRNA targeted to the first exon that is common to all transcript variants (Fig. 4A). The target site was chosen to be close to the beginning of the open reading frame in order that frameshift mutations produced would result in a non-functional protein.

Mutagenesis was monitored by immunostaining of cell populations with an anti-AGO1 antibody, which showed that upon transient transfection up to around 35% of cells contained homozygous null mutations in the AGO1 gene after 3 days. As expected, this proportion increased substantially upon selection, reaching a maximum of 82% mutant cells after selection for a further 7 days in puromycin (Fig. 4B,C; supplementary material Fig. S1). These results were further confirmed by sequence analysis of PCR products spanning the sgRNA target site, which showed that after transient expression, 39.1% of alleles had indel mutations at the expected cleavage site, which increased to 80.7% after selection in puromycin, consistent with the results from immunostaining (Fig. 4D). This suggests that selection for Cas9 expression with puromycin will allow the mutation of protein-coding genes at high efficiency. Further selection resulted in a loss of AGO1 mutant cells, and after 12 days of selection no
mutant cells were visible. This is likely due to a detrimental effect of AGO1 mutation on cell viability, resulting in wild-type cells outcompeting mutant cells within a population, since similar effects were not observed when Cas9 was expressed alone, or using a sgRNA targeting the yellow gene. It is also consistent with previous studies of RNAi-mediated knockdown of AGO1 levels, which show a highly reduced growth rate (Rehwinkel et al., 2006).

Tagging of the AGO1 gene
We further investigated whether homologous recombination could be used to insert an hemagglutinin (HA) epitope tag into the AGO1 protein, which could then be used to detect or purify endogenous AGO1 protein in the cell. Approximately 1 kb homology arms were designed to insert the HA sequence in frame with the AGO1 coding sequence, and our targeting construct was co-transfected with the AGO1 sgRNA/Cas9 vector. After transient transfection, around 1% of cells were observed to stain with an anti-HA antibody (Fig. 5A). This proportion increased upon selection in puromycin to a peak of approximately 4% (Fig. 5B).
observe staining of tagged cells with the anti-AGO1 antibody. This is likely due to epitope masking or deletion upon insertion of the tag. However, sequencing across the integration sites confirmed the expected homologous integration and tagging event.

**Discussion**

We have described an efficient system for generating genetic mutations in S2 cells that involves simple and rapid cloning of a pair of annealed 23–24 nt oligonucleotides, followed by transfection and selection to enrich for mutant populations. Efficiencies of up to 80% homozygous mutations in protein-coding genes could be achieved. This permits the analysis of genetic mutations in *Drosophila* cell lines for the first time. The approach can be used to assess the functionality of different sgRNAs before their use in *vivo*. Indeed, efficiencies of cleavage by different sgRNAs were found to differ substantially. This may be due to many reasons, including chromatin context of the target site, secondary structures within the sgRNA or the stability of sgRNA base-pairing with the DNA. Further studies with large numbers of sgRNA sequences will be necessary to define the rules of sgRNA design needed for efficient targeting.

Additionally we demonstrate the feasibility of homologous integration using relatively short 1 kb homology arms that are easy to generate and whose integrations are straightforward to detect by PCR. This approach could also be used to insert fluorescent or epitope tags into the endogenous copy of any gene, to enable affinity purification or detection of endogenous protein. We observe an efficiency of 1–4%, which is nevertheless sufficient to generate clonal populations either by serial dilution of cells (Nilsen and Castellino, 1999), or by using fluorescent activated cell sorting (FACS) to enrich for integration events such as GFP tagging of endogenous genes. It would also be possible to facilitate selection of homologous integrants by the incorporation of positive and negative selectable markers within and outside the homology arms in the targeting vector, as has been used in other systems (Huang et al., 2008; Mansour et al., 1988).

Upon selection in puromycin, it is probable that the non-integrated allele will be mutated due to indels created by imperfect NHEJ, hence the tagged protein will remain the only functional copy within the cell, which also enables the functionality of the tagged protein to be tested.

The application of the CRISPR/Cas9 system to *Drosophila* cell lines now also permits the construction of targeted deletions (Gratz et al., 2013; Kondo and Ueda, 2013; Ren et al., 2013) or insertions of recombinease sites to allow site-specific integrations (Groth et al., 2004). It also enables use of the various adaptations of the CRISPR/Cas9 system, which have been used to either activate (Maeder et al., 2013; Perez-Pinera et al., 2013) or repress transcription (Qi et al., 2013), to modify chromatin, or to recruit other protein or RNA molecules to specified DNA sequences.

The ease, speed and cost of generating sgRNA expression constructs should enable the production of genome-wide libraries of vectors that target the complete set of protein-coding genes using oligonucleotide printing technologies. If it is possible to screen or select for cellular phenotypes, for example using FACS or drug resistance gene expression, this should allow genome-wide screening of genetic mutants in S2 cells. The efficiency of mutagenesis should be sufficiently high to perform population-based phenotype measurements in a 96 or 384 well format, and to be complementary to currently available RNAi systems. This newfound ability to create stable genetic variants in *Drosophila* cell lines should now provide an adaptable and powerful system to study and screen for gene function at the cellular level.

**Materials and Methods**

**Vector construction**

A *Drosophila* U6 promoter followed by Bsr QI cloning sites and the remainder of the sgRNA backbone was generated as a GBlock (Integrated DNA Technologies), and cloned into the Bgl II site of the pAc-STABLE1-Puro vector (González et al., 2011). The human codon-optimised Cas9 was PCR amplified with Phusion DNA polymerase (New England Biolabs (NEB)) from pX330 (Mali et al., 2013b) and cloned into the *Bgl* II site of the pAc-sgRNA-STABLE1-Puro vector. Oligonucleotide sequences are indicated in supplementary material Table S1.

**sgRNA production and ligation**

sgRNA target sequences were selected as 20 nt sequences preceding an NGG PAM sequence in the genome. Overhangs were added to allow ligation into the vector. Two oligonucleotides for each target were synthesised and annealed by mixing 10 μl each oligonucleotide (100 μM) and 20 μl 2× annealing buffer (20 mM Tris, 2 mM EDTA, 100 mM NaCl, pH 8.0), and slowly cooling from 98°C to room temperature over a period of approximately 2 h. 1 μl of this mixture was phosphorylated in a 10 μl reaction with 1 μl T4 polynucleotide kinase (New England Biolabs), in T4 DNA ligase buffer at 37°C for 30 min and then diluted 10× in water. 2 μg pAc-sgRNA-Cas9 vector was digested with 20 U

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**Fig. 5. Epitope tagging of the AGO1 gene.** (A) Tagging of AGO1 with a HA epitope tag. Cells transfected with the AGO1 sgRNA and homology construct shown in Fig. 4A were analysed by immunostaining with anti-AGO1 (red) and anti-HA (green) antibodies and counterstained for DNA (cyan). Staining with anti-HA antibody is clearly observed in some cells (open arrowhead), with a cytoplasmic distribution similar to that of AGO1. Many cells show no AGO1 staining (closed arrowhead) due to homozygous mutation of the endogenous AGO1 gene. (B) Quantification of AGO1 tagging efficiency. Number of cells staining with anti-HA antibody were counted 3 days after transfection (transient), or after a further 4 or 7 days selection in puromycin (Selected 4 d or 7 d). Values were expressed as a percentage of the total number of cells (AGO1 wt and mutant), and error bars indicate 95% confidence intervals of at least 4 biological repeats of at least 200 cells per repeat. Scale bar: 10 μm.
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proteinase K at 55 °C for 2 h, followed by phenol:chloroform extraction and Coop for critical reading of the manuscript. Resource Centre (DGRC) for the S2R+ cell line, and Dr Sarah Further information concerning experimental methods and links to Acknowledgements

Estimation of homologous targeting efficiency DNA extractions were performed by incubation in lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5% N-lauroylsarcosine) with 200 μg/ml proteinase K at 55 °C for 2 h, followed by phenol:chloroform extraction and ethanol precipitation. PCRs across the homology arms and outside the integration site were performed with 100 ng DNA in 20 μl reactions and cycle numbers adjusted so that amplification was within the linear range. A standard curve consisting of a 2 fold serial dilution of each purified product was used to quantify amounts of each product, after quantification of gel images with Image J. Integration efficiency was calculated by comparison with PCR products outside of the homology arms. Sequencing of PCR products was performed after treatment with ExoSAP-IT (Affymetrix). Oligonucleotide sequences are shown in supplementary material Table S1.

High resolution melt analysis Oligonucleotides were designed to give 100–200 nt products spanning the presumed CRISPR cleavage site using Vector NIT (Invitrogen) (supplementary material Table S1). PCR was performed with Hotshot Diamond PCR mastermix (Clent Lifescience) in 10 μl reactions with cycle numbers adjusted so that amplification was within the linear range. A standard curve consisting of a 2 fold serial dilution of each purified product was used to quantify amounts of each product, after quantification of gel images with Image J. Integration efficiency was calculated by comparison with PCR products outside of the homology arms. Sequencing of PCR products was performed after treatment with ExoSAP-IT (Affymetrix). Oligonucleotide sequences are shown in supplementary material Table S1.

Immunofluorescence Approximately 1×10⁶ cells were transferred to 24 well plates containing circular coverslips, and left to settle for a minimum of 2 h. Cells were washed twice in 0.5 ml PBS, fixed in 0.5 ml 4% paraformaldehyde (Sigma) in PBS for 10 min and washed a further two times in 0.5 ml PBT (PBS + 0.1% Triton X-100). Blocking was performed in PBT (PBT + 5% normal horse serum) for 1 h, and primary antibodies added in PBTs overnight at 4 °C (anti-AGO1 (Soymi, 18B1) 1:1000, anti-HA (Roche, 3F10) 1:1000). Cells were washed 2×5 min in PBT, 1×10 min in PBTs and secondary antibodies added (λ mouse Cy5 1:1000, λ-rat Alexa 488 1:1000) for 4 h at room temperature in PBTs. Nuclei were stained with 10 μg/ml Hoescht 33342 for 10 min in PBT, and cells washed twice for 10 min in 0.5 ml PBT before mounting in Dako fluorescent mounting medium. Images were collected on a Leica SP5 confocal microscope, and images processed with LAS-AF software (Leica).

Acknowledgements Further information concerning experimental methods and links to discussion groups and other information are provided at the OXCRISPR website (http://groups.mrcfgu.ox.ac.uk/liu-group/useful-links/oxcrispr/oxcrispr). The expression plasmids described here will be made available from Addgene (Plasmids 49393: pAcsgRNA-Cas9 and 49331: pAcyl1sgRNA-Cas9). The authors would like to thank Dr Feng Zhang for plasmid pX330 (Addgene plasmid 42230 (Mali et al., 2013b), Dr James Sutherland for plasmid pSTABLE1-Puro (González et al., 2011), Dr Haruhiko Siomi for the anti-AGO1 antibody (Miyoshi et al., 2005), the Drosophila Genomic Resource Centre (DGRC) for the S2R+ cell line, and Dr Sarah Cooper for critical reading of the manuscript.

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Competing Interests The authors have no competing interests to declare.

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