ABSTRACT
Gene-editing techniques are revolutionizing the way we conduct genetics in many organisms. The CRISPR/Cas nucleases have emerged as a highly versatile, efficient and affordable tool for targeting chosen sites in the genome. Beyond its applications in established model organisms, CRISPR technology provides a platform for genetic intervention in a wide range of species, limited only by our ability to deliver it to cells and to select mutations efficiently. Here, we test the CRISPR technology in an emerging insect model and pest, the beetle Tribolium castaneum. We use simple assays to test CRISPR/Cas activity, we demonstrate efficient expression of guide RNAs and Cas9 from Tribolium U6 and hsp68 promoters and we test the efficiency of knockout and knock-in approaches in Tribolium. We find that 55–80% of injected individuals carry mutations (indels) generated by non-homologous end joining, including mosaic bi-allelic knockouts; 71–100% carry such mutations in their germ line and transmit them to the next generation. We show that CRISPR-mediated gene knockout of the Tribolium E-cadherin gene causes defects in dorsal closure, which is consistent with RNAi-induced phenotypes. Homology-directed knock-in of marker transgenes was observed in 14% of injected individuals and transmitted to the next generation by 6% of injected individuals. Previous work in Tribolium mapped a large number of transgene insertions associated with developmental phenotypes and enhancer traps. We present an efficient method for re-purposing these insertions, via CRISPR-mediated replacement of these transgenes by new constructs.

KEY WORDS: Gene editing, Insect, Evo-devo

INTRODUCTION
Until recently, gene targeting was a privilege of the few; it was possible only in a small number of organisms and involved sophisticated, labor-intensive techniques. Gene targeting in mammals required first modifying an allele in embryonic stem cells, then selecting the few cells carrying the targeting event and transplanting them into blastocysts to generate chimeras in which these cells would hopefully populate the germ line and contribute to the next generation (Capecchi, 2005; Smithies et al., 1985; Thomas et al., 1986). In Drosophila, after several failed efforts, efficient gene targeting was developed using a method that required bringing together three different transgenes (Rong and Golic, 2000). These techniques were not applicable to most other organisms, in which cultured pluripotent cells and sophisticated genetics were unavailable.

The invention of zinc-finger and transcription activator-like effector (TALE) nucleases marked a big step in our ability to target genes efficiently, by directing double-strand breaks to chosen sites in the genome and exploiting the cells’ endogenous DNA repair mechanisms to introduce changes at these sites (Carroll, 2014; Kim et al., 1996). Double-strand breaks are most frequently corrected by non-homologous end-joining (NHEJ) repair mechanisms, which can introduce small insertions or deletions (indels) at the site of repair. Less frequently, breaks are repaired through copying of a template that bears homologous sequences; such homology-directed repair (HDR) provides an opportunity to introduce specific changes into the locus via an engineered template. Zinc-finger and TALE nucleases proved to be extremely efficient for generating knockout and knock-in alleles compared with conventional gene targeting approaches. Their widespread use was limited mostly by the effort (and cost) required to customize their targeting specificity.

The recent discovery of CRISPR/Cas nucleases, whose sequence specificity is guided by simple base complementarity between the target DNA and a small guide RNA (gRNA), provided a simple, efficient and affordable way of customizing nuclease specificity (Jinek et al., 2012; Sander and Joung, 2014). CRISPR/Cas nucleases consist of protein and RNA. Their specificity is determined by base complementarity with the 5’ end of the gRNA followed by the presence of a ‘protospacer adjacent motif’ (PAM) in the target sequence. In the most commonly used CRISPR system, derived from Streptococcus pyogenes, the PAM sequence is NGG. Thus, by cloning the appropriate targeting sequence (N<sub>17</sub>-20) at the 5’ end of a gRNA, it is possible to generate nucleases targeting any sequence that conforms to N<sub>17</sub>-20NGG (where N can be any nucleotide).

Owing to this straightforward way of generating nucleases with a chosen sequence specificity and to its high targeting efficiency in a range of organisms, CRISPR technology holds great promise for emerging model organisms (Gilles and Averof, 2014). In principle, CRISPR-mediated gene targeting should be applicable to all organisms. In practice, the effectiveness of this approach is constrained by our ability to deliver CRISPR/Cas nucleases to cells of interest (e.g. to the germ line), by the nature and efficiency of the organism’s DNA repair mechanisms and by our ability to identify and maintain the resulting mutants. These parameters will ultimately determine the feasibility and efficiency of gene targeting in a given species.

Here, we present our effort to apply CRISPR technology in the beetle Tribolium castaneum and to establish methods and tools for efficient gene targeting in this species. Apart from being an important pest, infesting stored grain and grain products, Tribolium castaneum has emerged as an attractive experimental model for comparative developmental biology. Starting with classic genetic
screens conducted in the 1980s (Beeman et al., 1989; Sulston and Anderson, 1996), the Tribolium research community has grown, bringing a plethora of genetic tools and approaches to this species. These approaches include transgenesis (Berghammer et al., 1999, 2009; Lorenzen et al., 2003; Pavlopoulos et al., 2004), RNAi-mediated knockdown (Bucher et al., 2002; Posnien et al., 2009), enhancer trapping (Lorenzen et al., 2003; Trauner et al., 2009), heat-shock and GAL4/UAS-mediated mis-expression (Schinko et al., 2012, 2010) and live imaging (Benton et al., 2013; Sarrazin et al., 2012; Strobl and Stelzer, 2014). Moreover, the Tribolium genome is sequenced and well annotated (Kim et al., 2010; Richards et al., 2008). Based on these tools and resources, Tribolium castaneum has become the most advanced genetically tractable insect model after Drosophila.

Establishing efficient gene targeting in Tribolium brings to this model a more precise tool for reverse genetics, a directed approach for generating new markers and GAL4 drivers, and the opportunity to generate balancer chromosomes (for a more comprehensive discussion on the potential uses of CRISPR in emerging models, see Gilles and Averof, 2014).

RESULTS AND DISCUSSION
Targeting of a genomic EGFP insertion using CRISPR

We established an efficient assay for scoring gene targeting events in Tribolium, making use of the enhancer trap line Pig-19 (Lorenzen et al., 2003). Pig-19 is homozygous for a single insertion of pBac(3xP3-EGFP) in the Tribolium genome. Transgenic animals have strong enhanced green fluorescent protein (EGFP) fluorescence in larval, pupal and adult muscles (Fig. 1A,B). As a proof of concept for the functionality of CRISPR/Cas9 in Tribolium, we injected Pig-19 embryos with in vitro transcribed eGFP1 gRNA, targeting the EGFP coding sequence 116 bp after the start codon (Auer et al., 2014), together with capped Cas9 mRNA.

Three days after injection, the freshly hatched larvae were scrutinized for loss of EGFP expression in muscles. EGFP was visibly altered in 70-80% of the larvae (Table 1, rows 1, 2). The mildest phenotypes were small patches of non-fluorescent muscles, the strongest were loss of EGFP fluorescence in muscles of all segments (Fig. 1C). The loss of EGFP from muscles indicates biallelic targeting of EGFP in all the nuclei of the multinucleated muscle fiber, a sign of highly efficient somatic gene knockout.

To assess the frequency of germline targeting events, we raised injected (G0) animals and crossed them out to adult vermilion white beetles (Lorenzen et al., 2002). We then screened the progeny of each cross (G1) and determined the number of G0 animals that gave rise to non-fluorescent progeny (founders). Seventy-one percent of fertile crosses produced non-fluorescent progeny (Table 1, row 2). G0 animals showing mosaic loss of EGFP were more likely to produce non-fluorescent offspring (21 out of 22) compared with G0 animals with normal EGFP fluorescence (6 out of 16), suggesting that somatic targeting in G0 larvae may be a useful indicator of germline targeting in these animals.

The proportion of non-fluorescent larvae emerging from each cross was variable, ranging from 8% to 100% of G1 animals. Sequencing of the EGFP transgene of non-fluorescent

Fig. 1. Development of CRISPR-mediated gene targeting in Tribolium. (A) The Pig-19 transgenic line, used to test gene targeting, carries an insertion of 3xP3-EGFP which is activated by an endogenous muscle enhancer. The eGFP1 gRNA targets the EGFP coding sequence (red arrowhead). (B) Pig-19 larva showing strong EGFP fluorescence in muscles. (C) Pig-19 G0 larvae, arising from embryos injected with eGFP1 gRNA and Cas9 mRNA, showing mosaic loss of EGFP fluorescence. (D) Sequencing of the EGFP transgene from G1 larvae that lack fluorescence reveals small deletions at the eGFP1 target site (in gray). (E) Maps of the bhsp68-Cas9, U6a-Bsal-gRNA and U6b-Bsal-gRNA constructs, used to drive expression of gRNAs and Cas9, respectively. Detailed views of the U6a-Bsal-gRNA and U6b-Bsal-gRNA cloning sites are shown, indicating the sites where new targeting sequences can be inserted (green lines).
Cas9 transcribed gRNA and in vitro transgenes can be a useful alternative to delivering 2834 therefore set out to identify appropriate target sequence (reviewed by Gilles and Averof, 2014). We transcribe gRNAs also impose some additional constraints on the and robustness. The bacteriophage promoters used to in vitro Using promoters to express gRNA and Endogenous promoters to drive guide RNA and Cas9 expression

Using promoters to express gRNA and Cas9 from plasmids or transgenes can be a useful alternative to delivering in vitro transcribed gRNA and Cas9, in terms of ease of use, efficiency and robustness. The bacteriophage promoters used to in vitro transcribe gRNAs also impose some additional constraints on the target sequence (reviewed by Gilles and Averof, 2014). We therefore set out to identify appropriate Tribolium promoters.

First, we tested U6 promoters as a means of expressing gRNAs. U6 snRNAs are transcribed by RNA polymerase III, which is suitable for expressing small RNAs with precisely defined 5’ and 3’ ends, such as gRNAs (Cong et al., 2013; Dickinson et al., 2013; Jinek et al., 2013; Mali et al., 2013b; Ren et al., 2013). U6 promoters generate transcripts that start with a G and can thus be used to optimally target sequences that conform to G-N16-19-NGG (in practice, a mismatched G at the 5’ end of the gRNA can be tolerated; Fu et al., 2014; Hwang et al., 2013a).

We identified three U6 snRNA genes in the Tribolium genome. We cloned two putative promoter fragments, named U6a and U6b, spanning ~400 bp upstream of the transcription start site of two of those genes (see Materials and Methods). We placed these fragments upstream of the eGFP1 gRNA, generating plasmids p(U6a-eGFP1. gRNA) and p(U6b-eGFP1.gRNA), and tested their activity by injecting them into Pig-19 embryos together with Cas9 mRNA.

Overall, we found that the U6-gRNA plasmids gave similar targeting efficiencies and improved survival compared with in vitro synthesized gRNA (Table 1, rows 2-4). Somatic loss of EGFP in muscles was observed in 78% of G0 larvae. Knockout of EGFP was observed in the progeny of 91-100% of G0 animals with U6-driven gRNA versus 71% with in vitro gRNA (Table 1, rows 2-4). Thus, the Tribolium U6a and U6b promoters are capable of driving gRNA expression and mediating efficient gene targeting.

To facilitate the expression of different guide RNAs under these U6 promoters we designed plasmids p(U6a-BsaI-gRNA) and p(U6b-BsaI-gRNA), containing two BsaI restriction sites located 5’ of the gRNA scaffold. Complementary oligonucleotides carrying appropriate overhangs can be used to introduce the chosen targeting sequence in a single cloning step (see Fig. 1E; www.averof-lab.org/tools/Tribolium_CRISPR.php).

Next, we generated a Cas9 helper plasmid, p(bhsp68-Cas9), bearing the Streptococcus pyogenes Cas9 coding sequence downstream of the Tribolium hsp68 core promoter (Schinko et al., 2012), which drives low levels of expression constitutively. We tested the activity of this plasmid by co-injecting it with p(U6b-eGFP1.gRNA) in Pig-19 embryos. Sixty-two percent of G0 larvae showed somatic loss of EGFP in muscles, and 83% of outcrossed G0 animals produced non-fluorescent progeny (Table 1, row 5).

<table>
<thead>
<tr>
<th>gRNA source (ng/µl)</th>
<th>Cas9 source (ng/µl)</th>
<th>Knock-in template (ng/µl)</th>
<th>Number of embryos injected</th>
<th>Number of surviving larvae (%)</th>
<th>Number of mosaic knockout larvae (%)</th>
<th>Number of mosaic knock-in larvae (%)</th>
<th>Number of G0 animals crossed (%)</th>
<th>Number of knock-out founders (%)</th>
<th>Number of knock-in founders (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFP1 gRNA (30)</td>
<td>Cas9 mRNA (390)</td>
<td>–</td>
<td>562*</td>
<td>54 (10)*</td>
<td>43 (80)*</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>eGFP1 gRNA (30)</td>
<td>Cas9 mRNA (390)</td>
<td>–</td>
<td>441</td>
<td>55 (12)</td>
<td>39 (71)</td>
<td>–</td>
<td>38</td>
<td>27 (71)</td>
<td>–</td>
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<tr>
<td>p(U6a-eGFP1. gRNA)</td>
<td>Cas9 mRNA (180)</td>
<td>–</td>
<td>190</td>
<td>111 (58)</td>
<td>87 (78)</td>
<td>–</td>
<td>11</td>
<td>10 (91)</td>
<td>–</td>
</tr>
<tr>
<td>p(U6b-eGFP1. gRNA)</td>
<td>Cas9 mRNA (180)</td>
<td>–</td>
<td>295</td>
<td>123 (42)</td>
<td>97 (79)</td>
<td>–</td>
<td>17</td>
<td>17 (100)</td>
<td>–</td>
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<tr>
<td>p(U6b-eGFP1. gRNA)</td>
<td>p(hbsp68-Cas9) (1000)</td>
<td>–</td>
<td>166</td>
<td>34 (20)</td>
<td>21 (62)</td>
<td>–</td>
<td>18</td>
<td>15 (83)</td>
<td>–</td>
</tr>
<tr>
<td>p(U6b-eGFP1. gRNA)</td>
<td>p(hbsp68-Cas9) (500)</td>
<td>pBac(3xP3-DsRedfa) (500)</td>
<td>1866†</td>
<td>444 (24)†</td>
<td>246 (55)†</td>
<td>61 (14)†</td>
<td>315†</td>
<td>230 (73)†</td>
<td>18 (6)†</td>
</tr>
<tr>
<td>p(U6b-eGFP1. gRNA)</td>
<td>p(hbsp68-Cas9) (500)</td>
<td>pBac(3xP3-DsRedfa) (500)</td>
<td>1114†</td>
<td>242 (22)†</td>
<td>0 (0)§</td>
<td>0 (0)§</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>p(U6b-eGFP1. gRNA)</td>
<td>p(hbsp68-Cas9) (1000)</td>
<td>pBac(3xP3-DsRedfa) (500)</td>
<td>191</td>
<td>23 (12)</td>
<td>15 (65)</td>
<td>4 (17)</td>
<td>14</td>
<td>13 (93)</td>
<td>1 (7)</td>
</tr>
</tbody>
</table>

- ‡ Data from two independent experiments.
- § Data from five independent experiments.
- ¶ Data from four independent experiments.
- ¶ Co-injected with 200 ng/µl dsRNA for DNA ligase 4.

Table 1. Results of CRISPR experiments

In our hands, CRISPR/Cas9 performed more efficiently than a pair of zinc-finger nucleases that are known to target EGFP (Porteus, 2006): no somatic loss of EGFP was detected upon ZFN mRNA injection in Pig-19 embryos and 26% of these G0 animals (5 out of 19) produced non-fluorescent G1 progeny.

Endogenous promoters to drive guide RNA and Cas9 expression

Insert this text into the appropriate section of the document.
These results establish plasmid injection as an efficient means for the routine delivery of Cas9 and gRNAs in Tribolium. The relevant plasmids are available from www.addgene.com (EMBL/Genbank sequence accession numbers KR732918, KR732919 and KR732920).

**Targeting of endogenous loci**

To demonstrate targeting of endogenous loci in the Tribolium genome, we generated a gRNA targeting the coding sequence of *Tribolium E-cadherin* (gene TC013570) in plasmid p(U6b-Ecadherin.gRNA), as described above. We co-injected this plasmid with p(bhsp68-Cas9) in embryos of the vermilionwhite strain. Previous work had shown that embryonic RNAi for E-cadherin produces embryos that fail to complete dorsal closure, which can be observed in larval cuticle preps (A.F.G. and G. Bucher, unpublished). In our experiment, 53% (46 out of 86) of injected embryos developed a cuticle and 24% of those larvae (11 out of 46) showed dorsal closure defects (Fig. 2A,B). PCR amplification of the target locus from two G0 larvae revealed CRISPR-induced deletions at the target locus (Fig. 2C).

We performed similar experiments targeting the coding sequence of *Tribolium twist* and found that 99% of the G0 embryos (244 out of 247) failed to hatch. PCR amplification of the target locus from two G0 animals revealed several different mutations surrounding the target site (Fig. 2D).

**CRISPR-induced homology directed knock-ins**

Taking advantage of the Pig-19 line and the eGFP1 gRNA, we tested the efficiency of CRISPR in generating knock-ins in *Tribolium*. Specifically, we attempted to replace the 3xP3-EGFP transgene in the Pig-19 line by a 3xP3-DsRed transgene, exploiting the arms of the piggyBac transposon flanking the transgene to guide HDR; the homologous arms are 0.7 and 1 kb in length (Fig. 3A).

We injected p(U6b-eGFP1.gRNA) and p(bhsp68-Cas9) together with pBac(3xP3-DsRedaf) (Horn et al., 2002) as a template for HDR, and assessed EGFP and DsRed fluorescence in G0 and G1 larvae. To assess reproducibility, we repeated this experiment five times (the pooled results are shown in Table 1, row 6).

Two weeks after injection, somatic loss of EGFP was seen in 46-70% of G0 larvae and DsRed fluorescence in muscle fibers was observed in 8-20% of G0 larvae (Fig. 3B). Crossing the surviving G0 animals to vermilionwhite beetles, we found that 24-77% of crosses produced G0 larvae lacking EGFP expression and 0-10% of crosses produced larvae in which EGFP fluorescence was replaced by DsRed fluorescence in the muscles and in the 3xP3-driven pattern. DsRed-expressing G1 larvae were obtained in four out of five experiments. The proportion of DsRed-expressing larvae emerging from each cross ranged from 2% to 34%, among ~50 screened larvae. Sequencing the entire Pig-19 insertion locus from a G1 larva confirmed the seamless homology-driven replacement of the 3xP3-EGFP transgene by 3xP3-DsRed. No somatic targeting was seen in the absence of Cas9 (Table 1, row 7).

As noted for knockouts, somatic targeting in the G0 generation correlated with the likelihood of targeting in G1: across all five experiments, the fraction of G0 animals giving rise to DsRed-expressing G1 larvae was 12% among G0 animals with mosaic expression of DsRed (6 out of 50) versus 5% among non-DsRed-expressing G0 animals (12 out of 265).

Experiments in Drosophila show that mutations in DNA ligase 4, an enzyme required for NHEJ, can lead to an increase in the efficiency of HDR-mediated knock-ins (Beumer et al., 2008, 2013).
To test whether RNAi knockdown of DNA ligase 4 can improve knock-in efficiency in Tribolium, we performed the DsRed knock-in experiment with the addition of double-stranded RNA for Tribolium DNA ligase 4 in the injection mix. Knock-in efficiency was not significantly improved: 17% of G0 larvae showed mosaic expression of DsRed and 7% of crossed G0 animals produced DsRed-expressing G1 larvae (Table 1, row 8). Maternally deposited DNA ligase 4 protein, which is unaffected by embryonic RNA, may account for this result.

Excision and replacement of genomic regions

Using the same tools, we investigated the feasibility of deleting a specific genomic fragment by using pairs of double-strand breaks, and of replacing that fragment by an exogenous DNA construct. This approach could have numerous applications, such as deleting single exons or re-purposing transgenes (such as gene traps) at their original site of insertion.

To test the approach, we designed gRNAs targeting the left and the right arms of the piggyBac transposon (pBacL and pBacR). In the genome of Pig-19 beetles these sites delimit a fragment of ~2 kb that includes the 3xP3-EGFP transgene. The replacement transgene, 3xP3-DsRed, is flanked by the same target sites in plasmid pBacL (3xP3-DsRedaf) (Horn et al., 2002) and is therefore targeted by the same gRNAs (Fig. 4A). Plasmids expressing gRNAs for pBacL and pBacR were injected together with p(bhsp68-Cas9) and pBac(3xP3-DsRedaf). Forty-seven percent of the surviving G0 animals (22 out of 47) displayed mosaic DsRed fluorescence in muscles, sometimes accompanied by loss of EGFP, indicating integration of DsRed in the Pig-19 locus.

Thirty-four percent of G0 animals outcrossed to vermilionwhite beetles produced some progeny that had lost EGFP fluorescence. Of those, 22% G0 animals had some progeny in which the loss of EGFP was accompanied by DsRed fluorescence in muscles, indicating the replacement of the EGFP transgene by DsRed, and 12% G0 animals had some progeny that had both DsRed and EGFP fluorescence in muscles, presumably resulting from the insertion of the 3xP3-DsRed transgene at the pBacL or the pBacR target site without concomitant deletion of 3xP3-EGFP (Table 2).

To investigate the nature of these targeting events, we amplified and sequenced the genomic DNA of selected G1 animals at the Pig-19 insertion locus. Among the sequenced G1 animals that had lost EGFP fluorescence, six out of seven carried an excision of the 2-kb fragment delimited by the two gRNA target sites (Fig. 4B; the seventh carried a partial inversion and deletion of the transgene). All the G1 animals with DsRed fluorescence accompanied by loss of EGFP (4 out of 4) had replaced the 3xP3-EGFP transgene by 3xP3-DsRed, in the expected orientation (Fig. 4C). The integration events seamlessly restored the pBacL and pBacR sequence in six out of the eight insertion breakpoints, suggesting a precise repair by HDR; the other two breakpoints carried 3-bp deletions, which suggests that they were repaired by NHEJ. Some G1 larvae (5 out of 18) failed to produce the expected PCR bands, possibly reflecting more complex deletion and rearrangement events.

Conclusions

Our experiments demonstrate that CRISPR technology is an effective method for generating targeted knockouts, knock-ins and deletions in Tribolium. Deletion of genomic fragments driven by pairs of double-strand breaks, as demonstrated here, can be used to test the function of specific exons and putative cis-regulatory elements. Transgene replacement, shown for Pig-19, can be used to exploit the large collection of transgene insertions that already exist.
in *Tribolium* (Lorenzen et al., 2003; Trauner et al., 2009) to produce a wider range of genetic tools, such as GAL4 drivers (Schinko et al., 2010).

In this study, we did not investigate potential off-target effects of CRISPR. As in other experimental systems, we expect that unintended targets will occasionally be hit in the *Tribolium* genome (Cho et al., 2014; Fu et al., 2013; Hsu et al., 2013; Pattanayak et al., 2013). Equally, we anticipate that the technical solutions developed to reduce and control for these effects (discussed by Gilles and Averof, 2014) will also apply to *Tribolium*.

The promoters and constructs that we established for the expression of Cas9 and gRNAs facilitate the implementation of CRISPR in this species. We hope that these tools will also help to establish transgene-based applications of CRISPR, such as inducible and tissue-specific targeting (Port et al., 2014), and effectors for manipulating the function of genes in their native context (Mali et al., 2013a).

### MATERIALS AND METHODS

**Cas9 mRNA and gRNA synthesis**

Cas9 mRNA was *in vitro* transcribed from Addgene plasmid MLM3613 (Addgene; Hwang et al., 2013b) linearized with *PmeI*, using the

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**Table 2. Results of CRISPR-mediated excision and replacement experiments**

<table>
<thead>
<tr>
<th>gRNA and Cas9 injected (ng/μl)</th>
<th>Replacement template (ng/μl)</th>
<th>Number of embryos injected</th>
<th>Number of G0 animals crossed</th>
<th>Number of G0 founders (%)</th>
<th>Events scored among G1 animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>p(U6b-pBacL.gRNA) and p(U6b-pBacR: gRNA) (200 each), p(bhsp68-Cas9) (500)</td>
<td>pBacL(3xP3-DsRedaf) (250)</td>
<td>170</td>
<td>41</td>
<td>27 (66)</td>
<td>No targeting</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14 (34)*</td>
<td>EGFP excision</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9 (22)*</td>
<td>EGFP-to-DsRed replacement</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 (12)*</td>
<td>DsRed insertion</td>
</tr>
</tbody>
</table>

*Individual G0 founders produced different types of targeting events in G1 animals, so these categories overlap.
mMESSAGE mMACHINE T7 Ultra kit (Ambion). The eGFP1 guide RNA was in vitro transcribed from a plasmid (Auer et al., 2014) linearized with DraI, using the T7 Megascript kit (Ambion).

Cas9 helper plasmid

The Cas9 coding sequence was amplified from plasmid MLM3613 (Addgene; Hwang et al., 2013b) using primers AG004 and AG004e, which carry AgeI and NotI restriction sites (supplementary material Tables S1 and S2). The PCR product was cloned into the AgeI and NotI sites of pSfLa(bhs68-NLS-EFGP.hsp3’UTR)/fa (Schinko et al., 2012), replacing EFGP by the Cas9 coding sequence.

U6 promoter and gRNA plasmids

Three U6 snRNA sequences were identified in the Tribolium genome by sequence similarity with the Dro sophila U6 snRNA, using BLAST. We amplified ~400 bp of upstream sequences from two of these genes, designated U6a and U6b. Using overlapping PCR primers (supplementary material Table S1), we cloned each of these sequences upstream of the eGFP1 guide RNA (Auer et al., 2014) or of a gRNA scaffold with BsaI cloning sites for inserting new targeting sequences (Fig. 1E; Hwang et al., 2013b), in pBluescript-II-KS+ and pBME-Amp (BioMatik) plasmids, respectively (supplementary material Table S2). A U6 transcription terminator (Tn) was included at the 3’ end of the gRNA scaffold.

CRISPR target sites in the Tribolium twist and the E-Cadherin coding sequences were identified using the flyCRISPR target finder (http://tools.flycrispr.molbio.wisc.edu/targetFinder/; Gratz et al., 2014). Pairs of complementary oligonucleotides were designed for each target site, leaving appropriate overhangs for inserting these sequences in the BsaI site of p(U6b-BsaI-gRNA) (oligo design tool available at www.averof-lab.org/tools/Tribolium_CRISPR.php). The oligonucleotides were annealed and cloned into p(U6b-BsaI-gRNA) digested with BsaI.

Tribolium DNA ligase 4

The Tribolium DNA ligase 4 ortholog (gene TC012219) was identified by sequence similarity with Drosophila DNA ligase 4 protein, using BLAST. A 2-kb fragment containing the largest exon was cloned from genomic DNA, using primers AG001 and AG002 (supplementary material Table S1), into pGEM-T-Easy (Promega) and injected in supercoiled form.

We thank Martin Klingler for discussions on the application of CRISPR in Tribolium; Max Telford, Meriem Takari and Josh Coulcher for preliminary experiments using zinc-finger nucleases; Thomas Auer, Filippo del Bene and Marco Grillo for discussing targeting strategies; and Gregor Bucher for sharing Tribolium stocks.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl?doi=10.1242/dev.125054/-/DC1

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