VPS13A: shining light on its localization and function
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CHAPTER 4

CRISPR/Cas9 based genome editing of Drosophila for the generation of a Vps13 knockout mutant and Vps13-GFP flies

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CHAPTER 4

ABSTRACT

Drosophila melanogaster has been a very valuable model organism in genetic research for almost a century by now. Over time many different techniques were applied for genome engineering and mutagenesis that are often time consuming and laborious including ethyl methane sulfonate (EMS), X-ray radiation, transposable elements, zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). The CRISPR/Cas9 system is currently the most popular tool for targeted mutagenesis due to high efficiency, specificity and easy application.

Chorea-Acanthocytosis (ChAc) is a very rare neurodegenerative disease caused by mutations in the VPS13A gene and consecutive absence of the VPS13A protein. Patients present with different movement disabilities and deformed red blood cells called acanthocytes. Data on the function and localization of Vps13A and its orthologs in different organisms is still limited and mainly based on studies in unicellular organisms. Here we shortly discuss the most commonly used methods for mutagenesis in Drosophila and elaborately describe the successful application of the CRISPR/Cas9 technique to generate a Drosophila Vps13 knockout mutant and a Vps13-GFP fly line. Both lines will be used in the future to help elucidate the function and localization of Vps13 and to gain more insight in the underlying mechanisms of ChAc.

Keywords
CRISPR/Cas9, Genome editing, Drosophila melanogaster, Chorea-Acanthocytosis, Vps13, Knockout, Endogenous tagging
INTRODUCTION

Mutagenesis and genome engineering in *Drosophila melanogaster*

The fruit fly (*Drosophila melanogaster*) is a broadly studied and powerful model organism to study a wide variety of biological processes and human diseases. The availability of a very extensive genetic toolkit is one of the core strengths of *Drosophila* research. Chemical and insertional mutagenesis in *Drosophila* is applied often to disrupt and study gene function and many mutants were successfully uncovered by unbiased forward genetic screens after X-ray radiation\(^1\) or ethyl methanesulfonate (EMS) treatment\(^2\). EMS is a chemical mutagen which is easy to administer and induces random mutations in the DNA by nucleotide substitutions at a high frequency\(^3\,4\). Disadvantageous of EMS treatment is the laborious mapping of mutations and the creation of mosaic F\(_1\) flies that might not transfer the mutation to the next generation unless the germline is also mutant\(^2\). Radiation with X-rays was the first method used to induce mutagenesis and can cause chromosome breaks, deletions, transpositions and inversions\(^3\). Mutation frequency is lower compared to chemical mutagenesis but there is no risk of mosaic offspring as X-ray induced mutations are mainly double-stranded DNA breaks that do not cause mosaicism in the progeny\(^2\,4\).

Transposable elements

Another common technique for mutagenesis in *Drosophila* makes use of transposable elements (*P*-elements and *piggyBacs*). Transposable elements are pieces of DNA that are mobile in the genome and mutations can be created by either insertion of the transposable element or imprecise excision of the inserted transposable element which leads to a large deletion in the genome (reviewed by\(^5\)). The benefit of this technique compared to EMS is the reduced chance of secondary mutations in the genome. In addition, it is possible to combine the transposable element with a visible marker like *white*\(^+\) thereby making it easy to screen potential mutants based on absence or presence of the marker\(^6\). One drawback of using transposable elements for mutagenesis is that often the insertions are not accompanied by any phenotype due to the genomic location they are inserted\(^7\), but the major disadvantage of *P*-elements is their preference to insert in so-called “hotspots”. Although *piggyBac* elements have less insertional specificity and can therefore be used for more random insertion, their downside is that they only excise precisely and mutagenesis can only be reached via direct gene disruptions after the insertion\(^5\,6\). On the other hand, if direct insertion of a *piggyBac* element leads to gene disruption, excise precision of the element can be of benefit because it will create a suitable control for the observed phenotype and further functional studies.

Homologous recombination

The above mentioned mutagenesis techniques are all used to create random mutations that involve extensive screening (a so called forward genetic screen) of potential mutants and mapping of the induced mutation in the DNA. When the genome sequence of a gene of interest is known it is possible
to specifically mutagenize this gene via a reverse genetic approach. Homologous recombination (HR) is the classical technique used for targeted mutagenesis. HR repairs double strand breaks (DSBs) after DNA damage and normally occurs regularly at low frequencies. With the use of HR, mutations can be created either by disruption of the gene or by replacing an allele with a donor sequence. To make use of this HR repair mechanism, first a donor sequence has to be randomly inserted into the genome as a transgene. This donor sequence consists of a P-element with DNA homologous to the target site and a recognition site for a restriction enzyme. Later this donor sequence is excised from the DNA as circular DNA after which a DSB needs to be introduced via the restriction site to provoke homologous recombination with the complementary DNA of the "host" cell. Unfortunately those techniques suffer from low recombination frequency and many false-positives and are therefore not ideal for large screenings.

**ZFN and TALEN**

More recently, new techniques were developed to introduce a DSB in the genomic DNA and target basically any gene of interest. Different repair mechanisms in the cell are activated after introduction of a DSB. Since this repair is not always error-free it can lead to mutations in the targeted gene. Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have been successfully applied in *Drosophila* for targeted mutagenesis. Both ZFN and TALEN are chimeric enzymes that have a DNA binding domain and a non-specific DNA-cleavage domain. The DNA binding domain can be designed to match and target a specific region in the gene of interest. By combining multiple ZFN or TALEN domains it is possible to create a long and therefore more specific DNA recognition sequence. Every ZFN domain recognizes a triplet of nucleotides while an individual TALEN domain recognizes a single nucleotide, which provides greater design flexibility. The biggest drawback is that both ZFN and TALEN require the design and generation of a new and unique protein for every target and therefore this method is still not very suitable for large scale and systematic gene-targeting.

**CRISPR/Cas9**

Almost 30 years ago, clustered regularly interspaced short palindromic repeats (CRISPRs), were discovered in *E. coli*. Nowadays it is a very popular genome engineering technique that is applied in many model systems and organisms. In conjunction with a Cas protein, the CRISPR/Cas system functions as an adaptive immune system against foreign invasion of viruses and plasmids in many bacterial species. The best studied CRISPR/Cas system, from *Streptococcus pyogenes*, makes use of the Cas9 endonuclease and is now widely implemented as a genome editing technique. In this system a dual-RNA structure, the single-guide RNA (sgRNA), directs the Cas9 endonuclease to the DNA target site to create a double stranded break (DSB) (Figure 1A). The sgRNA consists of a CRISPR RNA (crRNA) module and a transactivating crRNA (tracrRNA) module. The crRNA provides specificity to the CRISPR/Cas9 complex by base pairing of a 20 nt targeting sequence that is complementary to the targeted DNA, while the tracrRNA module is required for the Cas9 nuclease activity. The only prerequisite for the system is that the target site of the sgRNA lies immediately 5' of a so-called PAM sequence. This 3 nt NGG protospacer-adjacent motif (PAM) is required for the DNA targeting. Once the DSB is induced by the CRISPR/Cas9 complex, this DSB can be repaired...
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by one of the endogenous DNA repair pathways: non-homologous end joining (NHEJ) or homology directed repair (HDR) (Figure 1B). Both of these repair pathways can be used to generate mutant alleles. NHEJ is an error-prone repair mechanism and can lead to localized small mutations due to deletion and/or insertion (indel mutations) of a couple of bp at the DSB-site22. These indel mutations can then result in null mutations or truncations of the protein sequence by shifting the reading frame of protein coding genes21,23. The HDR pathway allows more possibilities in genome editing and can introduce precise point mutations or insertions (knock-ins)21,24. For HDR it is necessary to supply a donor template that includes the desired insert flanked by two arms that are homologous to the target region23.

CRISPR/Cas9 in Drosophila melanogaster

CRISPR/Cas9 based gene editing has become a very popular method for targeted mutagenesis in Drosophila research because it has advantages over the other previously mentioned techniques. Firstly, it is enough to generate a 20 bp sgRNA which is sufficient for the Cas9 to function specifically, while for
ZFN and TALEN it is necessary to create individual proteins for specific gene targeting. In addition, NGG sequences that serve as PAM-sites are very abundant in the genome, providing many targeting areas. Second, it is possible to combine the Cas9 with a selection marker thereby simplifying the screening for potential mutants. It is also possible to combine the Cas9 with the Cre-loxP system to create conditional knock-out fly lines. Finally, multiple methods are available to deliver the CRISPR/Cas9 system to fly embryos, which gives researchers the option to select their method of preference. These methods include the injection of the CRISPR/Cas9 components as in vitro constructs (Cas9 mRNA and sgRNA) or as DNA plasmids, as well as the injection of a sgRNA DNA plasmid into Cas9 transgenic fly embryos and the establishment of sgRNA expressing transgenic fly lines that are crossed with a Cas9 transgenic fly line for mutagenesis to occur. Injection of a sgRNA plasmid into Cas9 transgenic embryos is considered to be the fastest, easiest and most cost-effective method to generate new mutant fly lines. Transgenic Cas9 fly lines are available with the construct under control of a general promoter for both the germline and somatic cells, or under the control of germline specific promoters like nanos and vasa. Since the Cas9 transgene is available on different chromosomes it is possible to out-cross the transgene after the generation of the desired lines.

**Chorea-Acanthocytosis and Vps13**

Chorea-Acanthocytosis (ChAc) is a rare neurodegenerative disorder caused by mutations in the vacuolar protein sorting 13 A (VPS13A) gene. Mutations in the VPS13A gene lead to the absence of the VPS13A protein, also called Chorein. The disease is characterized by progressive onset of hyperkinetic movements and the presence of deformed red blood cells with spike-like protrusions: acanthocytes. Recent research of Vonk et al. published data on the first multicellular model for ChAc (Chapter 3 of this thesis). This work supports previous data by discovering a role for *Drosophila melanogaster* Vps13 in autophagy and protein homeostasis. In this study a mutant fly line (*Vps13c03628*) was adopted from the Harvard Exelixis insertion collection that carries a P-element insertion in an intronic region of the Vps13 gene located in the center of the gene. Because of the P-element insertion site the *Vps13c03628* mutant is not a full Vps13 knock-out and a truncated Vps13 protein is still present. Potentially this truncated protein might have a dominant negative function and therefore the need arises to create a new mutant *Drosophila* model in which Vps13 is entirely absent. Furthermore, technical difficulties associated with the Vps13 gene made it impossible to rescue the *Vps13c03628* mutant by overexpression of a wild type transgene. Therefore it is of high importance to create an independent Vps13 mutant fly model that shows comparable phenotypes as the *Vps13c03628* mutant. In this case we can be most certain that those phenotypes are in both cases caused by the mutated Vps13 gene rather than them being the consequence of the genetic background unrelated to the Vps13 gene.

Here we aimed to generate a new *Drosophila melanogaster* mutant model for Chorea-Acanthocytosis with the use of the CRISPR/Cas9 system that can be used to support and extend the knowledge about the underlying disease mechanism of ChAc. In addition, we successfully applied the CRISPR/Cas9 system to endogenously tag the Vps13 gene and Vps13 protein with a Green Fluorescent Protein (GFP) reporter to investigate Vps13 localization. The creation of a Vps13 mutant in combination with a Vps13-CFP fly line
proved to be of high importance for especially the investigation of Vps13 localization in a multicellular organism (Chapter 5 of this thesis). In this methodology Chapter we elaborately describe the materials and methods for the generation and identification of a Vps13 knock out mutant and Vps13-GFP flies with the use of the CRISPR/Cas9 technology. The strategy can be adapted for every Drosophila gene of interest.

**MATERIALS AND METHODS**

**sgRNA design for Vps13 mutants and Vps13-GFP flies**

DSBs generated with CRISPR/Cas9 will be primarily repaired via NHEJ when a donor construct is absent, leading to indel mutations at the target site\(^3\). NHEJ is very effective to generate frame-shift mutations in coding sequences that lead to premature stop-codons\(^3\) and therefore is the preferred approach for gene disruption\(^3\). For the creation of the Vps13 mutants, two sgRNAs were designed and directed against the N-terminus of the Vps13 gene. With the use of two sgRNAs we aimed to generate a large defined deletion in the Vps13 gene giving rise to a full Vps13 knockout mutant\(^6\). In addition, this approach offered the possibility to create mutations caused by either one of the sgRNAs that result in a full Vps13 knockout mutant or possibly mutants with smaller indel mutations that do not lead to the complete absence of the Vps13 protein. For the generation of a Vps13-GFP line the sgRNA target was the final exon of Vps13 as close as possible to the gene terminator. All sgRNAs had to be 20 nt long and had to start with a G to optimize the U6-driven transcription of the sgRNAs\(^2\) and target sites should be followed by a 3-nt PAM sequence of NGG. SgRNAs were selected using the Optimal Target Finder of flyCRISPR (http://tools.flycrispr.molbio.wisc.edu/targetFinder/; retrieved in May 2015). For the generation of new Vps13 mutants we selected one sgRNA that targets exon 4 and a second sgRNA targeting exon 8 (Figure 2). Those sgRNAs met the previously mentioned requirements and had the highest quality score, which is determined by the inverse likelihood of off-target binding (tested on crispr.mit.edu). The sgRNA for the generation of the Vps13-GFP fly line was selected in the same manner.

**Figure 2. The Vps13 gene and sgRNA target sites.**

Schematic representation of the Vps13 gene and the sgRNA target sites for the generation of a Vps13 knockout mutant (orange) and Vps13-GFP flies (green). Exons are shown as grey boxes. Location of the PBac element of the current Vps13\(^{c03628}\) mutant is indicated (blue triangle). Vps13 protein and the epitope of the polyclonal C-terminal Vps13 antibody (#62) are depicted.
Cloning of sgRNA oligonucleotides into the pU6-BbsI-chiRNA plasmid

SgRNA targeting sequences are synthesized as oligonucleotides (Table 1) that are annealed and cloned into the pU6-BbsI-chiRNA plasmid (Addgene plasmid #45946; gift from Melissa Harrison, Kate O’Connor-Giles & Jill Wildonger) via BbSI restriction sites. Both oligonucleotides contain an overhang sequence that is complementary to the overhangs generated by BbsI digestion and should be 5’ phosphorylated. Annealing was done by increasing the temperature to 95°C followed by a slow cool down of the mixture by 5°C/minute in T4 ligation buffer (Figure 3). After digestion of the pU6-BbsI-chiRNA plasmid with the BbsI restriction enzyme the annealed oligos were ligated with the linearized plasmid using quick ligase and transformed into *E. coli*. Multiple colonies were sequenced to analyze correct insertion of the sgRNAs into the vector (data not shown) before injection.

Table 1. Primers sgRNA & donor plasmid

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<th>Name</th>
<th>Forward primer</th>
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<td>sgRNA exon 4</td>
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<tr>
<td>sgRNA exon 8</td>
<td>CTTCATAGTGACCCCAACCATAT</td>
<td>AAACCATATGCTGCCCATAC</td>
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<td>sgRNA GFP tag</td>
<td>CTTCATTAGACGCCCATACGCC</td>
<td>AAACCCCTACTCAGGCTAATAC</td>
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<td>5’ homologous arm HDR plasmid</td>
<td>GCCCTACGCCGCTGCCAATCCCTA</td>
<td>ATTACAGCCGCAAGTACCTCA</td>
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<tr>
<td>3’ homologous arm HDR plasmid</td>
<td>TGGCCTAGTGG</td>
<td>GCCCTTAAGGGATTG</td>
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<tr>
<td>GFP tag</td>
<td>CTATCCCCGCTATATGCACCAGCCTAC</td>
<td>GCCAAATAATTTCAGTTCATG</td>
</tr>
<tr>
<td>3’ homologous arm HDR plasmid</td>
<td>ACCCAAGGGCGACCCGCTTGCTCAC</td>
<td>GCCGCCTACTTACACCGCTCAGT</td>
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Donor plasmid design and cloning for Vps13-GFP flies

For the creation of the Vps13-GFP flies we used the Bluescript II SK+ plasmid (Stratagene) donor plasmid in which we cloned the GFP sequence flanked by two homologous arms. This assembly vector is injected together with the sgRNA and serves as a template for the homology directed repair after the DSB induction. To prepare the donor plasmid we linearized it by restriction digestion with NotI HF and EcoRV and then extracted it from agarose gel following electrophoresis. The inserted fragment consisted of three DNA fragments: a 5’ Vps13 homologous arm, a GFP fragment and a 3’ Vps13 homologous arm (Figure 4A, Table 1) that were PCR amplified individually. When designing the HDR fragments it is important to disrupt the sgRNA target site by changing the PAM sequence in the HDR construct. Introduction of a silent mutation in the PAM sequence that does not alter the amino acid sequence can be done by one or more nucleotide changes thereby preventing continuous cleavage by the Cas9 protein after the HDR construct is inserted in the genome. Both homologous arms, with a sequence immediately adjacent to the cleavage site of the sgRNA to facilitate efficient HDR, were generated with the use of a Bac clone (Pacman BAC collection, clone CH321-1b19, bapacresources.org), while for the GFP fragment we used a pEGFP-C1 (Clontech) plasmid as template. The primers for the PCR products were designed to overlap to be able to anneal the individual PCR products using the Gibson assembly reaction (Figure 4A,B). With the Gibson assembly it is possible to assemble multiple overlapping DNA fragments, regardless of fragment length or end compatibility in a single-tube isothermal reaction. We included the linearized Bluescript II SK+ vector in the Gibson reaction to assemble all independent PCR fragments and the vector in one
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**Figure 3. Cloning of sgRNA oligonucleotides into the pU6-BbsI-ChiRNA plasmid.**

Schematic representation of the pU6-BbsI-ChiRNA plasmid containing the U6 promotor sequence, two BbsI restriction sites, the tracrRNA sequence and the U6 terminator sequence. The target sequence of the sgRNA (crRNA guide sequence) is formed by annealing two oligonucleotides that both have an overhang sequence complementary to the overhangs that are created by BbsI digestion of the plasmid. After BbsI digestion of the plasmid the annealed oligonucleotides are ligated into the plasmid forming the sgRNA together with the tracrRNA sequence already present in the pU6-BbsI-ChiRNA plasmid.

**Figure 4. HDR plasmid design and Gibson reaction.**

(A) Primer design and DNA fragment size for assembly of the HDR plasmid digested with NotI and EcoRV (blue boxes) including a 5’ homologous arm of Vps13 (purple box), the GFP sequence (green box) and a 3’ homologous arm (yellow box) used for generation of Vps13-GFP flies. Primers create DNA fragments that have overlapping sequences to facilitate assembly of all DNA fragments with the Gibson reaction. (B) Schematic representation of the Gibson Assembly cloning method. Individual DNA fragments with overlapping ends are combined in three steps within one reaction: first the 5’ end of the DNA fragments are removed by an exonuclease creating complementary 3’ overhangs. Second the DNA fragments are annealed. Finally the 3’ ends are extended by a DNA polymerase that fills in gaps within each annealed fragment and the nicks of the assembled DNA are sealed by a DNA ligase. (Figure adapted from neb.com).
reaction after which the product was transformed directly into *E. coli*. Multiple colonies were investigated using restriction analysis for insertion of the product into the vector and correct donor plasmids were sequenced to confirm correct insertion of the PCR products into the vector (data not shown).

**Embryo injection and establishing stable lines**
For the generation of *Vps13* mutant and *Vps13-GFP* flies we chose to inject the sgRNAs and donor plasmid into embryos that express Cas9 only in the germline. The advantage of using embryos with Cas9 expression limited to the germline is that they are more reliable in producing heritable mutations because somatic mutations in the injected flies that can lead to lethality do not occur\(^{27,28}\). In addition, it was shown that transgenic expression of Cas9 in the germline increases mutagenesis efficiency and is more effective than coinjection of the Cas9 DNA/mRNA and sgRNA\(^{27,29}\). Embryo injection was performed by BestGene (Chino Hills, CA, United States) into *yw;;nos-Cas9(III-attP2)/Tm6C* embryos according to their protocol after which larvae were shipped back for further establishment of stable lines (Figure 5). Adults that developed from injected embryos were individually crossed to *if/CyO dfdYFP* balancer flies to obtain nonmosaic flies, after which we again crossed all F1 adults individually to *if/CyO dfdYFP* flies. Stable stocks were established from F2 flies (Figure 5).

![Figure 5. Embryo injection and crossing scheme to establish stable fly lines.](image)

Schematic representation for the generation of stable lines after embryo injection. sgRNAs were injected into nos-cas9 expressing embryos that developed into mosaic adult flies with male or female germline stem cells with or without mutations. Individual mosaic flies were crossed with a balancer stock, *if/CyO dfdYFP* in this case since *Vps13* is located on the second chromosome. Individual F1 flies were again crossed with the *if/CyO dfdYFP* stock. F2 flies were then used to establish a stable line by crossing male and female siblings.
Screening of stable lines

There are several methods available to detect genome alterations caused by CRISPR/Cas9. Screening can be done with F1 flies prior to establishing stocks. In order to do so, genomic DNA can be extracted from a single wing without killing the flies40 allowing selection of F1 mutants prior to individual crossing and establishing stable stocks22. Figure 6 shows a flow diagram of the screening process for both potential Vps13 mutants (Figure 6A) and potential Vps13-GFP lines (Figure 6B). One of the phenotypes of the current Vps13<sup>c03628</sup> mutant is male sterility. We hypothesized that the potential new Vps13 mutants would also show male sterility and therefore this was used as the initial screen. For the Vps13-GFP flies we did not want the GFP tag to functionally influence the Vps13 protein, and potential Vps13-GFP lines should therefore behave as wild type controls without male sterility or other phenotypes. Homozygous males from the stable lines were allowed to mate with w<sup>1118</sup> females for five days after which vials were analyzed for wandering larvae.

Figure 6. Screening methods for identifying Vps13 knockout mutants and Vps13-GFP flies.

Flow diagram of the used screening methods to identify Vps13 knockout mutants (purple) and Vps13-GFP flies (green). (A) After embryo injection and establishment of stable stocks potential Vps13 mutant lines were tested for male sterility and other homozygous phenotypes. Fly lines that showed male sterility or other homozygous phenotypes were then tested on Western blot to investigate Vps13 protein levels. In parallel, those same lines were crossed with the current Vps13<sup>c03628</sup> mutant and transheterozygous flies were tested for male sterility and the other observed homozygous phenotypes. The same lines that were tested on Western blot were further analyzed for presence of mutations by DNA sequencing across the cleavage sites of both sgRNAs. Finally, fly lines of which sequencing revealed a mutation in Vps13 were crossed with the mutated allele over a deficiency allele and scored for presence of their original phenotype. (B) Following injection and establishing of stable lines, potential Vps13-GFP lines were PCR screened for the GFP sequence. For a small selection of GFP positive lines we tested whether the GFP sequence was inserted in the proper genomic location through PCR screening for GFP and flanking regions with the use of primers directed against the genomic DNA upstream and downstream of the GFP insertion site. Next those lines were tested for male sterility followed by DNA sequencing to confirm proper insertion of the GFP sequence. Finally, expression of the Vps13-GFP protein was confirmed by Western blot.

In addition to male sterility, homozygous flies of all stocks were screened for the presence of additional visible phenotypes. Potential Vps13 mutant lines that showed homozygous male sterility and other phenotypes were further analyzed using Western blotting for the presence or absence of Vps13 protein and affected mutants were confirmed by DNA sequencing across the cleavage sites of both sgRNAs to determine the exact location and nature of the mutation (Figure 6A). In addition, those lines were crossed...
with the current Vps13\textsuperscript{C03628} mutant and transheterozygotes were again screened for male sterility and other phenotypes. The lines of which sequencing revealed a mutation in the Vps13 gene were eventually crossed with the mutated Vps13 allele over a deficiency line lacking a genomic region including the Vps13 gene and tested to see whether their original homozygous phenotype was still present.

For the potential Vps13-GFP lines we started with PCR amplification screening for the presence of GFP in the genome. Lines positive for GFP were then further analyzed by spanning PCR that amplifies the entire modified region to test whether the GFP was correctly inserted in the right genomic location at the C-terminus of the Vps13 gene. In addition, those lines were tested for male sterility. The identified Vps13-GFP lines were then sequenced and further confirmed using Western blot analysis (Figure 6B).

**Western blot analysis**

Western blot analysis was performed on samples of fly heads to check for Vps13 protein levels in potential mutant Vps13 fly lines. For the Vps13-GFP fly lines Western blot was used to confirm Vps13-GFP expression in lines that were already identified as Vps13-GFP using PCR and sequencing. In addition to Vps13, samples of potential Vps13-GFP lines were also tested using a GFP antibody. Flies were snap frozen in liquid nitrogen and decapitated by using a vortex. 4 µL of 2x Laemmli buffer (2% SDS, 10% glycerol, 0.004 % bromophenol blue, 0.0625 M Tris HCl pH 6.8) containing 0.8 M urea and 50 mM DTT was added per fly head. Samples were sonicated 5 times for 5 seconds and boiled for 5 minutes. Protein extracts were run on 8% polyacrylamide gels, transferred onto PVDF membranes overnight using transfer buffer containing 10% methanol. Membranes were blocked with 5% milk in PBS 0.1% Tween-20 and subsequently incubated with primary antibodies overnight at 4°C. The primary antibodies used were: rabbit anti-Vps13 #62 (1:1000, Vonk et al. 2017), rabbit anti-Vps13 NT (1:1000, Vonk et al. 2017), mouse anti-GFP (1:5000, Clontech), mouse anti-alpha-tubulin (1:5000, Sigma). Appropriate secondary HRP-conjugated antibody staining (1:5000, GE Healthcare) was done at room temperature in 5% milk for both Vps13 antibodies and PBS 0,1% Tween-20 for GFP and alpha-tubulin. Detection was performed using ECL or super-ECL solution (Thermo Scientific) with the ChemiDoc Touch (BioRad).

**Genomic DNA isolation and PCR screening Vps13-GFP flies**

To isolate Drosophila genomic DNA (gDNA) of potential Vps13-GFP flies, two different protocols were used:

1) Two flies of the potential Vps13-GFP lines were placed in an eppendorf tube containing 100 µL squishing buffer (10mM Tris-HCl pH 8.2, 1 mM EDTA, 25 mM NaCl and 400µg/ml proteinase K) and mashed for 20-30 seconds. After 30 minutes incubation at 37°C the proteinase K was inactivated by heating the samples to 95°C for 3 minutes. The samples were centrifuged shortly and the supernatant was used for PCR.

2) Five flies of the potential Vps13-GFP lines were collected and mashed for 20-30 seconds using a yellow pipet tip with 50 µL solution A (0.1 M TrisHCl pH 9.0, 0.1 M EDTA and 1% SDS). Samples were incubated at 70°C for 30 minutes after which 7 µL 8M KAc (Merck) was added per sample and followed by incubation
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on ice for 30 minutes. The samples were centrifuged for 15 minutes at 13,000 rpm at 4°C after which the supernatant was transferred to a fresh tube. 30 µL of isopropanol (Sigma Aldrich) was added to each sample and after some shaking the samples were centrifuged for 5 minutes at 10,000 rpm at 4°C. Afterwards the supernatant was removed and the pellet was washed with 70% EtOH and centrifuged for 5 minutes at 13,000 rpm. After removing the supernatant, the pellet was dried to open air. Finally the pellet was resuspended in 20 µL RNAse and DNase free H2O (Life Technologies).

The DNA sequences of the potential Vps13-GFP lines were initially screened for presence of GFP (gDNA isolation protocol 1) using the “GFP” primers listed in Table 2. A small selection of lines positive for GFP were then further analyzed by PCR for the flanking regions of the GFP sequence to check whether the GFP was fused to the 3’ end of the Vps13 gene (gDNA isolation protocol 2) using the “GFP + flanking regions” primers listed in Table 2. DNA sequences were amplified using Paq5000 Hotstart PCR Master Mix (Agilent). After amplification the samples were run on an 0.8% agarose gel and visualized with the Chemidoc MP System (Bio-Rad).

Sequencing of potential Vps13 mutants

Genomic sequencing was performed for potential mutant flies that showed absence of Vps13 protein on Western blot and for fly lines with additional homozygous phenotypes. Genomic DNA was isolated using a TRIZOL method: 25 embryos or 8 L3 larvae were collected and snap frozen in liquid nitrogen. The samples were homogenized in 250 µL of TRIZOL (Invitrogen) with a motor pestle and incubated at room temperature for five minutes. Afterwards 100 µL of chloroform was added and tubes were shaken vigorously by hand or vortex and incubated at room temperature for 2-3 minutes. Samples were centrifuged at 12,000 rpm for 15 minutes at 4°C. The aqueous phase was transferred to a fresh RNase/DNase tube and stored for potential RNA analysis. For the DNA extraction we used TNES-6U containing 10 mM Tris-HCl, pH 7.5, 125 mM NaCl, 10 mM EDTA pH 8.0, 1% SDS, 6M Urea. To fully dissolve the Urea the solution was pre-heated to 40°C (40°C TNES-6U). Per sample 75 µL of the 40°C TNES-6U was added to the organic phase and interphase and samples were mixed by gentle shaking and incubated at room temperature for 10 minutes. Then samples were centrifuged for 15 minutes at 13,000 rpm at 4°C. About 100 µL of the aqueous phase was transferred to a clean tube and the interphase and organic phase were discarded. An equal volume of isopropyl alcohol was added to the aqueous phase per sample followed by incubation at -80°C for 2 hours. After the samples were thawed they were centrifuged at 13,000 rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet was washed twice by resuspending it in 70% EtOH followed by 3 minutes incubation and 5 minutes centrifugation at 13,000 rpm. All EtOH was removed and the pellet was dried to open air. Finally the pellet was re-dissolved in 50 µL low EDTA TE buffer (10 mM Tris, 0.1 mM EDTA pH 8.0) that was preheated at 65°C and the samples were incubated at 65°C for 15 minutes.

Table 2. Primers PCR Vps13-GFP

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<tr>
<th>Name</th>
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<td>AAGGGCGAGGAGCTGTTCAC</td>
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<td>GFP + flanking regions</td>
<td>CCTTCTTCGAGCCGCGTAT</td>
<td>GAAGTTCGTGACCAGGCCA</td>
</tr>
</tbody>
</table>

Notes:
Genomic regions surrounding the sgRNA target sites in exon 4 and exon 8 were PCR amplified using Phusion (New England Biolabs) and additional DMSO in the reaction mixture and sent for sequencing using the primers listed in Table 3.

**Sequencing of identified Vps13-GFP line**

Genomic DNA extraction for sequencing of the identified Vps13-GFP line and corresponding control was performed using protocol 2. Sequencing was done using the primer listed in Table 3.

**Table 3. Sequencing primers**

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seq. pU6-BbsI-chiRNA</td>
<td>TAATACGACTCACTATAGG (T7 primer)</td>
<td>-</td>
</tr>
<tr>
<td>Seq. pBluescript II SK+</td>
<td>GATACCGACACCCGTGAT</td>
<td>ATGCTTCCCGCTGTATGGG</td>
</tr>
<tr>
<td>Seq. GFP</td>
<td>GATGGTCGCCATCTTGG</td>
<td>CGGATGGGCACCACCC</td>
</tr>
<tr>
<td>Exon 4</td>
<td>CGAGCCGAAATGATGACG</td>
<td>-</td>
</tr>
<tr>
<td>Exon 8</td>
<td>TGCCGCATGCGCCCTCC</td>
<td>-</td>
</tr>
<tr>
<td>Vps13-GFP</td>
<td>TATAATGCAGCGCGAC</td>
<td>-</td>
</tr>
</tbody>
</table>

**RESULTS**

**Generation and identification of a Vps13 knockout mutant**

We aimed to establish a Vps13 knockout mutant line for the further investigation of Vps13 function and localization. For the generation of a Vps13 knockout mutant more than 300 nos-cas9 embryos were injected with two sgRNAs targeting either exon 4 or exon 8 of the Vps13 gene. Out of the embryo injection we got 10 fertile mosaic flies (male or female) that gave rise to 282 independent stable lines (Suppl. Table 1). Most of the stable lines did not show any abnormalities, however in some occasions different homozygous phenotypes were observed among those lines in different stages of development including embryonal and pupal lethality, rough eyes and held-out wings (Figure 7).

In addition, multiple lines appeared to be male sterile when tested. Table 4 lists the lines that showed any of the before mentioned phenotypes that were further investigated and tested on Western blot. Each line was given a number which consists of the number of the original mosaic precursor line followed by a specific number for the individual stable line. We also selected one stable line per mosaic progenitor that did not show any phenotype to serve as internal control, with the exception of progenitor 14, that only gave rise to stable lines that showed one or the other phenotype (held-out wings or male sterility). Progenitor line 14 and 22 gave rise to many lines with comparable phenotypes, we only selected 3 individual lines per phenotype for further investigations. To examine whether the observed phenotypes are accompanied by the absence of Vps13 protein all lines were tested using western blot (Figure 8; Table 4).
Generation of Vps13null and Vps13-GFP flies using CRISPR/Cas9

Figure 7. Drosophila life cycle and observed homozygous phenotypes during different stages of development of potential Vps13 knockout lines.

Schematic representation of Drosophila development that consists of the following stages: embryogenesis, three larval stages, a pupal stage and the adult stage. Screening of stable lines that were established after injection of the two sgRNAs for the generation of a Vps13 knockout mutant revealed several phenotypes during different stages of development including embryonal lethality, pupal lethality, rough eyes, held-out wings and male sterility.

Figure 8. Vps13 protein levels of potential Vps13 knockout lines.

Samples from homozygous fly heads of selected fly lines with homozygous phenotypes were analyzed by Western blot for Vps13 levels using the C-terminal Vps13 #62 antibody. WT118 was used as a positive control while the Vps13<sup>c03628</sup> mutant served as a negative control. α-Tubulin was used as a loading control.
Table 4. Overview of selected lines with homozygous phenotypes and their controls.

<table>
<thead>
<tr>
<th>Line number</th>
<th>Phenotype</th>
<th>Transheterozygous phenotype</th>
<th>Protein</th>
<th>Sequence abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-2</td>
<td>(Control)</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4-4</td>
<td>♀ sterile</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4-28</td>
<td>Pupal lethal</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8-1</td>
<td>(Control)</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8-2</td>
<td>Pupal lethal</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8-6</td>
<td>Pupal lethal</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10-3</td>
<td>(Control)</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10-36</td>
<td>Pupal lethal</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10-39</td>
<td>Pupal lethal</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14-3</td>
<td>Held-out wings</td>
<td>Yes</td>
<td>6 bp deletion + bp missense exon 4</td>
<td></td>
</tr>
<tr>
<td>14-6</td>
<td>♀ sterile</td>
<td>No 1 bp deletion exon 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-7</td>
<td>Held-out wings</td>
<td>Yes</td>
<td>6 bp deletion + bp missense exon 4</td>
<td></td>
</tr>
<tr>
<td>14-11</td>
<td>♀ sterile</td>
<td>No 1 bp deletion exon 4</td>
<td></td>
<td></td>
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<tr>
<td>14-16</td>
<td>Held-out wings</td>
<td>Yes</td>
<td>6 bp deletion + bp missense exon 4</td>
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</tr>
<tr>
<td>14-27</td>
<td>♀ sterile</td>
<td>No 1 bp deletion exon 4</td>
<td></td>
<td></td>
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<tr>
<td>22-1</td>
<td>Embryonal lethal</td>
<td>No</td>
<td>2 bp deletion exon 8</td>
<td></td>
</tr>
<tr>
<td>22-2</td>
<td>Embryonal lethal</td>
<td>Yes</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>22-7</td>
<td>♀ sterile</td>
<td>No 2 bp deletion exon 8</td>
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<td></td>
</tr>
<tr>
<td>22-8</td>
<td>(Control)</td>
<td>Yes</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>22-39</td>
<td>♀ sterile</td>
<td>No 2 bp deletion exon 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23-1</td>
<td>(Control)</td>
<td>Yes</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>23-17</td>
<td>Rough eyes</td>
<td>Yes</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>23-18</td>
<td>Rough eyes</td>
<td>Yes</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>42-1</td>
<td>(Control)</td>
<td>Yes</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>42-17</td>
<td>Pupal lethal</td>
<td>Yes</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Since extracts of adult fly heads were used for the analysis, lines that showed homozygous lethality, either during embryonal stage or pupal stage, were crossed with the current Vps13<sup>C03628</sup> mutant that shows no protein when detected using the C-terminal antibody (Vps13 #62), and transheterozygous flies were analyzed to enable analysis of adult fly heads. Although there is some variability between the lines, it is surprising that many lines with homozygous phenotypes still show presence of Vps13 protein. For some of the lines, Vps13 protein levels were below detection limit, making them interesting candidates for new Vps13 knockout mutants. Because of the presence of Vps13 protein in the other lines it is unlikely that those observed phenotypes are caused by reduced Vps13 levels. It is however still possible that potential mutations do not affect Vps13 protein levels but do interfere with normal function. To investigate whether the sgRNAs induced genomic mutations the DNA from all selected lines and from both targeted loci was amplified and sequenced (Table 4). Most of the stable lines accompanied by a homozygous phenotype like embryonal or pupal lethality and rough eyes, but without the absence of Vps13 protein, did not show any sequence abnormalities in either exon 4 or exon 8. One exception are the flies with the held-out wing phenotype (line 14-3, 14-7 and 14-16), that have a mutation in exon 4 consisting of the deletion of 6 base pairs (bp) followed by a missense mutation of one bp. Because this mutation does not lead to a DNA frameshift, the Vps13 gene is probably still transcribed and translated properly which explains why the protein can still be detected. As those three stable lines are all originating from the same mosaic
Generation of Vps13null and Vps13-GFP flies using CRISPR/Cas9

Precursor fly, and the mutation is the same for all three lines they probably were derived from the same germline stem cell. Even though those three fly lines show the homozygous phenotype of held-out wings and have a mutation in the Vps13 gene, combining their mutated allele with a deficiency line abrogated their held-out wing phenotype which indicates that the phenotype is caused by an off-target mutation rather than the mutation found in Vps13. Sequencing revealed that the selected male sterile lines from mosaic precursor number 14 (14-6, 14-11 and 14-27), of which protein levels are below detection limit, all have the same mutation in exon 4 (one bp deletion). Those lines still show a male sterile phenotype as transheterozygotes with the current Vps13<sup>c03628</sup> mutant as well as when their mutated allele is crossed over a deficiency line.

The male sterile lines that originate from mosaic precursor number 22 (22-7 and 22-39) both have a deletion of two base pairs in exon 8 that leads to the absence of Vps13 protein visualized by Western blotting. Combining those mutated alleles with either the current Vps13<sup>c03628</sup> mutant or with the deficiency line maintains the male sterility, and therefore line 22-7 and 22-39 can be considered as bona fide Vps13 knockout mutants. Besides those two male sterile lines, the embryonal lethal line 22-1 shows the same mutation. Interestingly, when this line is combined with either the Vps13<sup>c03628</sup> mutant or with the deficiency line the embryonal lethality is lost, but transheterozygous males are sterile. This suggests that the deletion of the two base pairs found in exon 8 leads to male sterility and that the embryonal lethality is caused by an off-target mutation induced in the genome at an unknown position. This is further supported by the data of line 22-2, which shows the embryonal lethality as well, but this is not accompanied by the absence of Vps13 protein and no mutation was found in the Vps13 gene when this line was sequenced. In addition, transheterozygotes of line 22-2 with either the Vps13<sup>c03628</sup> mutant or the deficiency line do not show any phenotypes.

Taken together, many of the lines we selected based on their homozygous phenotype are not accompanied by a mutation in the Vps13 allele and absence of Vps13 protein. Overall germline transmission rate, which is the number of mutant offspring divided by the total number of stable lines, is 10%. The lines 14-6, 14-11, 14-27, 22-7 and 22-39 can be considered new Vps13 knockout mutants caused by a mutation in either exon 4 or exon 8 leading to the absence of Vps13 protein and male sterility. Mosaic progenitor 14 did not produce stable lines containing an intact Vps13 gene, so mutant lines derived from this progenitor cannot be compared to a proper control line with the same genetic background and similar treatment. For line 22-7 and 22-39 this is possible since line 22-8 does not have any mutations or phenotypes and can therefore serve as the proper control for future experiments.

**Generation and identification of a Vps13-GFP fly line**

Next to generating a Vps13 knockout mutant, we aimed to establish a fly line that endogenously expresses Vps13-GFP to further investigate Vps13 localization. To generate such a Vps13-GFP line the sgRNA and HDR donor plasmid were simultaneously injected into ~300 nos-cas9 embryos after which the larvae were shipped to our lab and we established stable lines as described in the materials & methods section. Genomic DNA of almost 70 stable lines (which were numbered in the same way as for potential Vps13 mutant lines, but with the addition of a ‘g’ to indicate they are potential GFP lines) was isolated (protocol 1
in Material and Methods) after which a PCR amplification screening for the presence of GFP in the genome was performed (Figure 9). This PCR revealed that many of the lines we tested were positive for GFP.

Next we wanted to further investigate the GFP positive lines and analyze whether the GFP sequence was inserted in the right location at the C-terminus of the Vps13 gene by using primers upstream and downstream of the GFP in the genomic DNA. We randomly selected 12 lines derived from two precursor flies (g10 and g16) of which we extracted genomic DNA using a different protocol to continue the PCR screening (protocol 2 in Material and Methods). Since the primers are directed against the genomic DNA upstream and downstream of the preferred GFP insertion site, both control and GFP-tagged Vps13 gave a PCR product (Figure 10A). Correct insertion of the GFP sequence however leads to the addition of 750 bp to the PCR product. Figure 10 shows that two lines do not contain GFP-tagged Vps13 (g10-21 and g16-7), but multiple lines do (g10-4; g10-22; g10-25; g16-2; g16-27 and g16-28) based on PCR product size. Male sterility tests with those lines showed no abnormalities in offspring and males were fully fertile which suggests that the fused Vps13-GFP protein is still functional, although protein function has to be investigated further. Finally we selected line g16-28 as potential Vps13-GFP line with g16-7 as internal control and sequencing of line g16-28 revealed the proper insertion of the GFP sequence at the selected site downstream of Vps13 while the genomic sequence of line g16-7 was not different from wild type (data not shown). Western blot analysis further confirmed the presence of the Vps13-GFP protein in line g16-28 (Figure 10B). This line is potentially of high importance to study the endogenous localization of Vps13.
DISCUSSION

Our results demonstrate that the CRISPR/Cas9 technique can be employed to create precisely targeted mutations in the *Drosophila melanogaster* Vps13 gene, thereby generating a Vps13 knockout mutant. In addition, we successfully applied the technique to genetically fuse the Vps13 gene with a fluorescent GFP tag by providing a donor construct and making use of the homology directed repair pathway. To generate a Vps13 knockout mutant we aimed to increase the efficiency of mutagenesis by simultaneous injection of two sgRNAs with target sequences in the N-terminus of the Vps13 gene, as generation of mutations and defined deletions using two sgRNAs was shown to increase the mutagenesis efficiency. However, the efficiency and overall germline transmission rate for mutations in Vps13 was relatively low: 10% of which 6% actually has a mutation that leads to absence of the Vps13 protein. Accessibility of the target site, secondary structures within the sgRNA and thermodynamic stability of the sgRNA-DNA duplex were all suggested to influence cleavage efficiency. In addition, CG content of sgRNAs, and mainly of the six most proximal nucleotides to the PAM sequence, affects mutagenesis efficiency with sgRNAs containing four or more CGs in this region giving rise to a high heritable mutation rate. Carefully selecting the most efficient sgRNA based on CG content will most likely improve the efficiency in future studies.

Figure 10. Identification of a Vps13-GFP fly line.

(A) PCR amplification of the GFP sequence and flanking genomic regions was performed on a small selection of potential Vps13-GFP lines that were GFP positive. Primers were designed upstream and downstream of the preferred GFP insertion site at the C-terminus of the Vps13 gene to confirm proper insertion of the GFP sequence. Without GFP the primers give a product of 2820 bp (Vps13, black triangle) and correct insertion of GFP gives a PCR product of 3570 bp (Vps13-GFP, open triangle). Samples containing heterozygous flies will show both bands. (B) Extracts of control (g16-7) and the identified Vps13-GFP line g16-28 fly heads were analyzed by Western blot to confirm presence of Vps13-GFP protein using a GFP antibody. Vps13 #62 antibody was used to demonstrate presence of Vps13 protein and α-tubulin was used as a loading control.
Target genes of mutagenesis efficiency studies that were published were limited to *white*, *vermillion*, *ebony*, *rosy* and *yellow* with overall germline transmission rates up to 100%, although there is a high variation between studies and experiments and it remains difficult to predict sgRNA efficiency\(^{39}\). It might be that some mutations in *Vps13* went unnoticed due to our screening method. For example, flies that presented with held-out wings appeared to have a deletion in exon 4, but by combining the mutated allele with a deficiency line the held-out wing phenotype was lost indicating an off-target effect being responsible for the observed phenotype. Since the mutation in *Vps13* did not lead to the absence of *Vps13* protein and male sterility it illustrates that mutations in *Vps13* can occur without influencing previously reported phenotypes of *Vps13* dysfunction. This shows that those mutations might have been overlooked as they do not come up in the initial screening method of male sterility thereby underestimating the overall germline transmission rate. Based on the described experiments we cannot exactly determine the overall germline transmission rate because some of the crosses were affected by fungal growth in the food that might have led to the absence of viable offspring.

To our surprise we did not identify any mutant that had a defined deletion between the target sites of both injected sgRNAs while previously it was shown that simultaneous injection of two sgRNAs targeting the *white* gene significantly improved the overall heritable mutation rate\(^{27}\). It is possible that the creation of two DSBs at a relatively large distance in the genome (~1,5 kb) leads to chromosomal rearrangements that have detrimental effects on development and are often lethal\(^{41}\). This might have reduced the overall germline transmission rate in our study.

Next to efficiency, specificity of the CRISPR/Cas9 system is of high importance when applying this technique. Research of Fu *et al.*\(^{42}\) in three different mammalian cell types showed that sgRNAs can efficiently induce DSBs at off-target sites that have up to five mismatches compared to the original on-target sites. Mutagenesis efficiency on those off-target sites can even exceed efficiency at the original on-target site\(^{42}\). Although off-target cleavage sites are larger concern when the CRISPR-technique is applied to mammalian and other systems compared to *Drosophila* because of its relatively small genome size\(^{23,27}\), our data show that also in *Drosophila* off-target effects do occur. Most of the homozygous phenotypes we found were not caused by mutations in the *Vps13* gene but rather by an unidentified off-target cleavage. It is therefore crucial to properly control for off-target effects by combining potentially mutated alleles with deficiency lines and always confirm mutations by genomic sequencing of the target region.

*In vivo* tagging of endogenous genes can be of high importance for studying endogenous protein expression, localization and function. By making use of HDR induced by the CRISPR/Cas9 system it is possible to attain precise genomic insertions, which includes the *in vivo* tagging of a protein of interest with a fluorescent marker like GFP\(^{39,43}\). Here we showed that by simultaneous injection of a sgRNA and a donor plasmid that contains the *GFP* sequence flanked by two homologous arms into Cas9-expressing embryos it is possible to precisely insert the *GFP* before the stop codon of *Vps13*, thereby creating a fly line that expresses *Vps13*-GFP at the endogenous level. Flies expressing the *Vps13*-GFP gene did not show male sterility and therefore we predict that the protein is functional. It is however still important to further validate functionality and exclude other phenotypes.
Both the Vps13 knockout mutant and the Vps13-GFP line will be used to gain more insight in the function and localization of Vps13. Since the localization of Vps13 has never been demonstrated in a multicellular organism, this will be a helpful tool to elucidate the role of Vps13.

ACKNOWLEDGEMENTS

We thank Liza Lahaye for advise during the design phase of this study.
REFERENCES


34. Vonk, J. J. et al. Drosophila Vps13 is Required for Protein
Generation of Vps13null and Vps13-GFP flies using CRISPR/Cas9

Supplementary Table 1. Overview of the CRISPR/Cas9 treated embryos, larvae and flies for the generation of a Vps13 knockout mutant

<table>
<thead>
<tr>
<th></th>
<th># Injected embryos</th>
<th># Surviving larvae</th>
<th># Crosses</th>
<th># Fertile crosses</th>
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<td>Embryo injection</td>
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<td>Larval count</td>
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<td>F1</td>
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Generation of Vps13null and Vps13-GFP flies using CRISPR/Cas9