Non-Mendelian Dominant Maternal Effects Caused by CRISPR/Cas9 Transgenic Components in Drosophila melanogaster

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ABSTRACT The CRISPR/Cas9 system has revolutionized genomic editing. The Cas9 endonuclease targets DNA via an experimentally determined guide RNA (gRNA). This results in a double-strand break at the target site. We generated transgenic Drosophila melanogaster in which the CRISPR/Cas9 system was used to target a GAL4 transgene in vivo. To our surprise, progeny whose genomes did not contain CRISPR/Cas9 components were still capable of mutating GAL4 sequences. We demonstrate this effect was caused by maternal deposition of Cas9 and gRNAs into the embryo, leading to extensive GAL4 mutations in both somatic and germline tissues. This serves as a cautionary observation on the effects of maternal contributions when conducting experiments using genomically encoded CRISPR/Cas9 components. These results also highlight a mode of artificial inheritance in which maternal contributions of DNA editing components lead to transmissible mutant defects even in animals whose genomes lack the editing components. We suggest calling this a dominant maternal effect to reflect it is caused by the gain of maternally contributed products. Models of CRISPR-mediated gene drive will need to incorporate dominant maternal effects in order to accurately predict the efficiency and dynamics of gene drive in a population.

KEYWORDS gene drive maternal effect HACK mutagenic chain reaction MCR

The phenotype of a developing animal is not determined solely by the chromosomes inherited from each parent. The female gamete provides the early cytoplasmic environment crucial for the developing embryo (Bate and Arias 1993). This cytoplasm contains various organelles, RNA, and proteins as determined by the mother’s genomic and mitochondrial DNA which are essential for proper development. This maternal effect on development was highlighted in saturating mutational screens in Drosophila aimed at identifying genes required for normal development (Nüsslein-Volhard and Wieschaus 1980). Mutant mothers, even when crossed to a nonmutant father, produced embryos exhibiting profound defects, such as in the establishment of the body axes during early embryonic development (Perrimon et al. 1984; Schüpbach and Wieschaus 1986; Schüpbach and Wieschaus 1991). Products of these maternal effect genes (RNA and protein) are deposited into the egg where they play direct roles in the development of a fertilized embryo. Maternal effect inheritance is defined that all progeny from a mutant mother will show a mutant phenotype, even if the developing embryo contains a functional gene inherited from the father (Nüsslein-Volhard and Wieschaus 1980).

Gene editing techniques have been revolutionized in recent years by the introduction of the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system (Jinek et al. 2012; Bassett et al. 2013; Cong et al. 2013; Gratz et al. 2013; Mali et al. 2013; Doudna and Sontheimer 2014). This system, originally used in bacteria as a native defense mechanism against viral infection, utilizes the Cas9 class of proteins to generate a double-strand DNA break. The binding of Cas9 to DNA is directed by a 20 nucleotide guide RNA (gRNA), whose only restriction is the presence of a protospacer adjacent motif (PAM) sequence (Jinek et al. 2012; Mali et al. 2013). Given the adaptability of
MATERIALS AND METHODS

Drosophila genetics

The fly stocks used in the study are 5xUAS-GFPnls (BS#4775), 5xQUAS-nucLacZ (Potter et al. 2010) (BS#30006), 10xQUAS-6xGFP (Shearin et al. 2014) (BS#52264), 5xUAS-mtdt-3HA (Potter et al. 2010), Act5C-Cas9 (Port et al. 2014) (BS#54390), GMR57C10-GAL4 (BS#39171), GMR57C10-QF2cas (BS#66479), and attP2-QF2cas (negative orientation) (Lin and Potter 2016) (BS#66504).

Nervous system dissection and immunohistochemistry

Dissection of the adult Drosophila nervous system was performed as described previously (Lin et al. 2015). Brains of 4–6 d-old flies were dissected in 1× PBS solution and then fixed in 4% PFA solution (dissolved in PBS with 0.3% Triton X-100) for 15 min. Fixed tissues were washed briefly three times with PBT (PBS with 0.3% Triton X-100) before incubating in PBT for 20 min for two times. Next, 5% NGS (normal goat serum dissolved in PBT) was used as blocking solution for 30-min washing. The tissues were then placed in primary antibody mixes for 1–2 d, followed by PBT washes for 20 min for two times. The tissues were placed in secondary antibody mixes for 1 d in the dark. The following day the tissues were washed in PBT for 20 min and placed in mounting solution (Slow Fade Gold) for at least 1 hr at room temperature before imaging. All previous steps were performed at room temperature.

Immunostaining was used to enhance fluorescence signals. For GFP, the primary antibodies were rabbit anti-GFP (1:250, #A11122; Life Technologies) and chicken anti-GFP (1:1000, #GFP1020; Aves Labs Inc.). Secondary antibodies were Alexa 488 anti-rabbit (1:200, #A11034; Invitrogen) and Alexa 488 anti-chicken (1:200, #A11039; Invitrogen). For the nuclear LacZ staining, the primary antibody was preabsorbed rabbit anti-LacZ (1:50, #559761; MP Biochemicals). The secondary antibody was Alexa 649 anti-rabbit (1:200, #DI-1649; Vector Laboratories). To avoid the conflicting red colors from UAS-mtdt-3HA and 3xP3-RFP (transgene marker), the mtdt-3HA was counterstained with rat anti-HA (1:250, #11867423001; Roche) as the primary antibody and Alexa 633 anti-rat (1:200, #A21094; Invitrogen) as the secondary antibody. In order to visualize the structure of the nervous system, mouse anti-nc82 (1:25, Developmental Studies Hybridoma Bank (DSHB)) was used as the primary antibody and Cy3 anti-mouse (1:200, #115-165-166; Jackson ImmunoResearch) as the secondary antibody.

Confocal imaging and image processing

Confocal images were taken on a Zeiss LSM 710 confocal microscope equipped with 10x (Zeiss, Fluor 10×0.5) and 40x (Zeiss, Plan-Apochromat 40×/1.3 Oil DIC) objectives. Zen 2012 software was used for image acquisition. For illustration purposes, Z-stack images were collapsed onto a single image by ImageJ using maximum-intensity projection and pseudocolored into different acquisition channels using an RGB plug-in.

Cell counting

Image analysis was performed using ImageJ 1.5 (National Institutes of Health). A total of 3–4 single sections of 5-μm thick brain confocal images were collapsed into a single image, followed by color inversion (edit/invert) and threshold adjustment (image/adjust/threshold; setting to black & white). The processed image was then used for automated counting (analyze/analyze particle). The same procedures were performed for successive 3–4 sections until the whole tissue had been analyzed. Counts were summated to provide an estimated final count of labeled cells for the sample.

Data availability

All Drosophila lines are available at the Bloomington Drosophila Stock Center or upon request.
RESULTS AND DISCUSSION

An unusual maternal inheritance was identified in experiments using the recently developed HACK technique (Lin and Potter 2016), in which gRNA's are supplied with a donor transgene compatible for homology directed repair (HDR) with a genomically targeted location (Figure 1). In the GAL4 > QF2 HACK variant (QF2G4H donor), the method expresses gRNAs that target GAL4 sequences for double-strand breaks, along with a donor transgene containing GAL4 homology arms flanking a T2A-QF2 cassette (Figure 1A). In the presence of a ubiquitous Cas9 transgene (Actin5C-Cas9), GAL4 transgenes in the germline were “HACKed” to produce progeny expressing functional T2A-QF2 instead of functional GAL4. Upon a double-strand break triggered by the HACK system, GAL4 sequence could be disrupted by non-homologous end joining (NHEJ) or converted into QF2G4H by HDR (Figure 1B). Interestingly, since CRISPR/Cas9 components were expressed ubiquitously (Actin5C driving Cas9 and the RNA polymerase III promoter U6 driving gRNAs), genomic HACKing
could take place in somatic cells as well (Figure 1C). To visualize the status of GAL4 in individual cells, a dual reporter system was incorporated into all HACK genetic crosses (Figure S1). By using UAS-GFPnls (green fluorescent reporter) and QUAS-nucLacZ (stained with far-red fluorescent dye and pseudocolored red) to monitor the activity of GAL4 and QF2, respectively, we could distinguish intact GAL4 (Figure 1C, green), disrupted GAL4 (dGAL4, Figure 1D, no color), and QF2G4H (Figure 1E, red). Therefore, the activity of GAL4 or QF2 to drive UAS or QUAS reporter expression could be used as a proxy to monitor the activity of CRISPR/Cas9 genomic editing on somatic tissues encoding GAL4.

To determine the effectiveness of somatic HACKing in the nervous system, we used a pan-neuronal GAL4 line (GMR57C10-GAL4) as the target of Cas9 and the attP2-QF2G4H donor line (Lin and Potter 2016) (Figure 2). The adult Drosophila brain is estimated to contain ~100,000 neurons (Chiang et al. 2011). We observed very few nuclear GFP-labeled neurons (GAL4, UAS-GFPnls, 20.6 ± 2.97, n = 5) and ~0.8% of neurons labeled with nuclear LacZ (QF2G4H > QUAS-nucLacZ, 800 ± 55, n = 5) in the adult brains. These results indicated that the genomic GAL4 sequences of most neurons were disrupted into dGAL4 through NHEJ, and some neurons had GAL4 sequences HACKed into T2A-QF2 (Figure 2, A1 and B1). Disruption of GAL4 required the CRISPR/Cas9 components to be present in the genome (Figure 2, A2–A4).

Strikingly, this was not the case if the parental cross was simply reversed and mothers now contained Act5C-Cas9 and U6-gRNAs (Figure 2B and Figure S1). We found progeny with disrupted GAL4 transgenes even when U6-gRNAs, Act5C-Cas9, or both were absent in the offspring genome (Figure 2, B2–B4). For example, in the complete absence of genetically encoded CRISPR/Cas9 components, >90% of somatic neurons still contained dGAL4 (GFP cell count = 810 ± 174, n = 2) (Figure 2B4). Since the maternal genome contains both CRISPR/Cas9 components, we reasoned that offspring somatic GAL4 genes were targeted by maternally contributed gRNAs and Cas9 endonuclease present in the female gamete (egg). This is supported by the observation that GFP can be deposited into embryos by a maternal Act5C-GFP transgene (Reichhart and Ferrandon 1998). To directly verify the presence of Cas9 protein in the egg, we performed anti-Cas9 embryo immunostaining at early developmental stages (0–2 hr after egg laying). Indeed, Cas9 protein was observed only in embryos when parental crosses contained a maternal Act5C-Cas9 transgene, but not in those in which the Act5C-Cas9 transgene originated from the paternal side (Figure S2).
The development of the Drosophila oocyte is arrested twice during meiosis: prophase I and metaphase I (Bate and Arias 1993). Once activated and ovulated, the oocyte completes meiosis, finalizing the zygotic genotype, and continues with differentiation and development. Given the short period between meiosis completion and zygote cell division, it is likely that residual gRNAs and Cas9 proteins (and mature Cas9 mRNA) remain functional and generate double-strand breaks at the target GAL4 sequence in the dividing progenitor cells. The extent of GAL4 disruptions from maternal contributions was enhanced by the presence of CRISPR/Cas9 transgenes in the progeny genome, which would continue to supplement gRNA and/or Cas9 endonuclease during development (Figure 2, B2 and B3). The HACK system is compatible with maternal CRISPR/Cas9 contributions such that if only QF2G4H donor (but not the Cas9 transgene) is present in the zygotic genome (e.g., attP2-QF2G4H), HDR-mediated gene conversion of GAL4 to QF2G4H can occur (Figure 2, B1 and B3).

Drosophila germline stem cells are derived from the pole cells, which are the first cellularized structures in the syncytial embryo (Zalokar and Erk 1976; Foe and Alberts 1983). Pole cells inherit the maternal cytoplasm deposited at the pole plasma (Houston and King 2000). The maternally contributed gRNAs and Cas9 proteins could affect germline stem cells if these components were not excluded from the pole cells. To examine the status of GAL4 transgenes of F1 male germline cells (sperm), we crossed F1 males to females containing membrane-targeted GAL4 and QF2 reporters (UAS-mdt3HA and QUAS-6xGFP), and calculated the percentage of progeny containing functional GAL4, dGAL4, and functional QF2 (Figure 3 and Figure S1). Strikingly, even in the absence of CRISPR/Cas9 components in the genome, GAL4 disruption frequency was high (87.2%, n = 413 F2 flies, Figure 3). This suggests that maternal Cas9 and gRNA are incorporated in the pole cells, and are highly effective at targeting both somatic and germ lines. As expected, maximal frequency of GAL4 disruption was observed when there was a continuous supply of gRNA and Cas9 protein from the genome (98.2%, n = 164 F2 males, Figure 3). If gRNAs or Cas9 transgenes are lacking in the genome, the disruption frequencies fall in between (92% and 97%). Interestingly, a continuous supply of gRNA (vs. Cas9) appears to be more critical in disrupting GAL4, because more intact GAL4 individuals were observed when U6-gRNAs was absent in the F2 genome (Figure 3, bar 2 and 3). This could be the result of a shorter half-life of RNA (gRNA) compared to protein (Cas9). Furthermore, Cas9 protein could be supplemented by the translation of preloaded mature Cas9 mRNA transcripts. Finally, consistent with previous observations in somatic cells (Figure 2, B1 and B3), HACK gene conversion events were observed when QF2G4H donor sequence was present in the genome (Figure 3, bar 1 and 3).

These data demonstrate a form of non-Mendelian inheritance caused by artificial genetic engineering. A maternal effect is defined as the causal influence of the maternal genotype (or phenotype) on the offspring phenotype (Wolf and Wade 2009). In the case reported here, the maternally contributed CRISPR/Cas9 DNA editing components are directly influencing the offspring genotype, which in turn leads to changes in phenotype. Maternal effect mutant phenotypes typically are caused by a lack of maternally contributed RNA or proteins, whereas in this case, effects were caused by the gain of maternally contributed RNA and proteins. As such, we propose calling this form of inheritance a dominant maternal effect.

MCR-directed gene drive is considered a potential approach for the extermination or control of pest insects, such as the disease transmitting Aedes aegypti and Anopheles gambiae mosquitoes (Evett et al. 2014; Gantz et al. 2015; Hammond et al. 2015). The MCR technique uses the CRISPR/Cas9 system to target and replace a genomic locus with a self-replicating variant containing homology arms flanking Cas9 and gRNA cassettes (Gantz and Bier 2015). By HDR, the variant can copy itself into the wild-type sister chromosome in the genome. It can form the basis of artificial gene drive, whereby a mutant allele is introduced and spread throughout a population. As previously speculated (Port et al. 2014; Gantz et al. 2015), our results show that CRISPR/Cas9 components will be maternally deposited during oogenesis, and thus could lead to continued CRISPR/Cas9-directed effects even in the absence of genomically supplied DNA editing components. This would not pose a problem if the MCR-directed gene drive success rate were 100%, as the genomic template and CRISPR/Cas9 components would be perfectly coincident. However, since MCR requires HDR in the absence of NHEJ, the actual efficiency ranges from 91 to 99% (Gantz and Bier 2015; Gantz et al. 2015; Hammond et al. 2015). Our previous work indicated that HDR vs. NHEJ efficiency could be as low as ~10%, depending on the genomic location (e.g., attP2 locus) (Lin and Potter 2016). This results in mutation of the CRISPR target sites and the generation of heterozygous gene drive–resistant individuals in the progeny (Figure 4, light gray box). Furthermore, the maternal dominant effect highlighted in this work could function to promote the introduction of gene drive–resistant (R*) alleles into the population.
This would be most notable when maternally contributed Cas9 and gRNAs are still present but an MCR-compatible Cas9-gRNA cassette is not present in the progeny genome (Figure 4, red box). A homozygous gene drive-resistant individual could be potentially generated from heterozygous gene drive-resistant parents in the natural environment, but the probability would be low if the population density of heterozygous gene drive individuals is also low. The dominant maternal effect described here provides a short-cut that could directly generate homozygous gene drive-resistant individuals when gene drive is not successful in females (Figure 4 and Figure S3). The consequence is potentially rapid generation of homozygous gene drive-resistant animals in the population (Figure 4, dark gray box). Depending on the mechanisms of gene drive to control the population, gene drive-resistant individuals may gain a survival advantage given selective pressure, and counteract MCR-directed gene drive in a population (Gantz et al. 2015). These results suggest the most effective gene drive mechanisms will likely depend on supplying CRISPR/Cas9 genomic components only from the father, or simultaneously targeting multiple loci in a gene. Furthermore, models of CRISPR-mediated gene drive (Hammond et al. 2015) would need to incorporate dominant maternal effects as an inhibitory mechanism.

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