© 2021. Published by The Company of Biologists Ltd | Development (2021) 148, dev198010. doi:10.1242/dev.198010

TECHNIQUES AND RESOURCES

RESEARCH REPORT

A transgenic system for targeted ablation of reproductive and maternal-effect genes

Sylvain Bertho[1,*], Odelya Kaufman[2,*], KathyAnn Lee[1], Adrian Santos-Ledo[2], Daniel Dellal[1] and Florence L. Marlow[1,2,‡]

ABSTRACT

Maternally provided gene products regulate the earliest events of embryonic life, including formation of the oocyte that will develop into an egg, and eventually into an embryo. Forward genetic screens have provided invaluable insights into the molecular regulation of embryonic development, including the essential contributions of some genes whose products must be provided to the transcriptionally silent early embryo for normal embryogenesis, called maternal-effect genes. However, other maternal-effect genes are not accessible due to their essential zygotic functions during embryonic development. Identifying these regulators is essential to fill the large gaps in our understanding of the mechanisms and molecular pathways contributing to fertility and to maternally regulated developmental processes. To identify these maternal factors, it is necessary to bypass the earlier requirement for these genes so that their potential later functions can be investigated. Here, we report reverse genetic systems to identify genes with essential roles in zebrafish reproductive and maternal-effect processes. As proof of principle and to assess the efficiency and robustness of mutagenesis, we used these transgenic systems to disrupt two genes with known maternal-effect functions: *kif5ba* and *bucky ball*.

KEY WORDS: Mutagenesis, Maternal effect, Germ cells

INTRODUCTION

Maternally provided gene products regulate the earliest events of embryonic life, including formation of the oocyte, egg and eventually the embryo. Disruption of oocyte development or early embryogenesis causes congenital anomalies and are apparent in 2-5% of human births according to the National Institute of Child Health and Human Development. Chromosomal aneuploidy underlies some birth defects, but the genetic basis for others remains poorly defined (Ambartsumyan and Clark, 2008; Hassold et al., 2007). The most devastating mutations disrupt early embryogenesis when the diverse cell types necessary to build an embryo and the basic body plan are forming. Mutations occurring in later development are detectable in the children born with the consequent congenital deviations. In contrast, mutations in genes essential for processes occurring before implantation or during gastrulation result in embryonic lethality (Ambartsumyan and Clark, 2008; Hassold et al., 2007). In mammals, embryonic development occurs *in utero*, so mutations disrupting essential regulators of early embryogenesis often go undetected due to arrest *in utero* and miscarriage (Hassold et al., 2007; Hassold and Hunt, 2007; Zhao et al., 2006). Consequently, our understanding of the molecular and genetic regulation of this extremely sensitive developmental period remains incomplete.

In fish and humans, the earliest developmental events are regulated by maternally supplied gene products because the early zygote is transcriptionally silent (Abrams et al., 2020; Marlow, 2010; Sato et al., 2019; Vastenhouw et al., 2019; Wu and Vastenhouw, 2020; Zhao et al., 2006). These maternally supplied genes are known as maternal-effect genes because the embryo relies on gene function supplied by its mother. Mutations disrupting these genes in the mother affect the progeny regardless of the genotype of the embryo. Where studied, the basic aspects of oocyte development are remarkably conserved among animals, including regulation of meiotic initiation and arrest (Marlow, 2010). A number of maternal-effect genes have been discovered in the mouse; however, the precise contribution of maternal-effect genes is often masked by *in utero* arrest of the embryos (Marlow, 2010; Wu and Dean, 2020; Zhao et al., 2006). That is, although the gene is essential, the phenotype detected is lack of or underrepresentation of genotypically mutant progeny (if any) of mutant mothers, which hinders determining the specific cause of embryonic arrest. The external fertilization and development of zebrafish, and the large numbers of progeny produced weekly allow the recovery and examination of every egg produced, and the determination of the cellular and molecular basis of the developmental disruption.

Forward genetic screens have provided invaluable insights into the molecular regulation of embryonic development, including contributions of some maternal-effect genes (Dosch et al., 2004; Pelegri et al., 2004; Pelegri and Mullins, 2004; Wagner et al., 2004). However, maternal-effect genes with additional essential zygotic functions during embryogenesis are missed because the mutants do not reach reproductive maturity. Indeed, although the zebrafish is an excellent genetic system, traditional mutagenesis strategies and modern reverse genetic approaches alone have not permitted straightforward identification of maternal functions of zygotic lethals (Doyon et al., 2008; Foley et al., 2009a,b; Lawson and Wolfe, 2011; Moens et al., 2008; Sander et al., 2011a,b; Sood et al., 2006). To identify these factors, one needs to bypass the early zygotic requirement of the gene so that potential reproductive or maternal functions can be investigated. Methods to circumvent zygotic lethal phenotypes in the zebrafish were pioneered in the Schier and Raz labs (Ciruna et al., 2002). Their germline replacement approach takes advantage of early separation of somatic and germline lineages to generate animals with normal somatic composition and mutant germlines through host germline ablation and transplantation (replacement) with mutant germ cells

[1]Department of Cell, Developmental and Regenerative Medicine, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place Box 1020 New York, NY 10029-6574, USA. [2]Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx NY 10461, USA.
*These authors contributed equally to this work

‡Author for correspondence (florence.marlow@mssm.edu)

F.L.M., 0000-0002-9362-0342; F.L.M., 0000-0002-9362-0342

Handling Editor: Stephen Wilson
Received 23 October 2020; Accepted 21 May 2021

Development (2021) 148, dev198010. doi:10.1242/dev.198010

(Ciruna et al., 2002). This strategy allows the animal to survive to produce mutant gametes, which can be examined for reproductive and maternal-effect phenotypes. Although this approach has been applied to examine the function of specific genes (Bennett et al., 2007; Borovina et al., 2010; Ciruna et al., 2002, 2006; Williams et al., 2018), thus far, no systematic germline replacement screen of zebrafish zygotic lethal mutations has been attempted because this approach is challenging and inefficient.

One drawback of *dead end*-mediated germline replacement is that few females are produced (Ciruna et al., 2006, 2002). This clearly impedes studies of oocyte development or of maternal-effect functions. This male bias is in part due to insufficient numbers of donor PGCs to support female-specific gonadogenesis. Consequently, *dead end* morphant embryos become sterile males (Ciruna et al., 2002; Siegfried and Nusslein-Volhard, 2008; Slanchev et al., 2005; Weidinger et al., 2003). Additional evidence suggests that signals from oocytes support female gonadogenesis (Bertho et al., 2021; Cao et al., 2019; Dranow et al., 2016, 2013; Hartung et al., 2014; Kaufman et al., 2018; Rodriguez-Mari et al., 2010; Rodriguez-Mari and Postlethwait, 2011, 2011; Romano et al., 2020; Wu et al., 2020). Specifically, diminished oocyte numbers or stages result in masculinization and female to male sex reversal (Kaufman et al., 2018; Rodriguez-Mari et al., 2010, 2011; Rodriguez-Mari and Postlethwait, 2011; Romano et al., 2020; Wu et al., 2020). To circumvent this problem, we used a transgenic mutagenesis approach to generate mosaic gonads in which germ cells carrying mutagenic cassettes and potential mutations are marked with fluorescent reporters.

Here, we report reverse genetic systems to identify genes with reproductive and maternal-effect functions. This approach will be particularly useful for genes whose maternal-effect functions are masked by earlier zygotic roles in embryogenesis. This transgenic approach selectively mutates the germline and thus allows the animal to survive to produce mutant gametes, which can be examined for reproductive or maternal-effect phenotypes. As proof of concept, we used this system to disrupt two genes with known reproductive and maternal-effect phenotypes, *kinesin 1* (*kif5ba*) and *bucky ball* (*buc*). The potential to examine the function of every gene in its genome makes the zebrafish an extremely powerful vertebrate system to unravel molecular and genetic control of developmental processes and of adult physiology and disease. Complete phenotypic characterization of the zebrafish phenome will significantly improve our understanding of processes that are difficult to access in mammals, in particular maternal-effect processes.

RESULTS AND DISCUSSION

Vector based system to generate germline and maternal-effect mutants

Traditional mutagenesis strategies and modern reverse genetic approaches alone have only provided limited access to zebrafish maternal-effect genes (Doyon et al., 2008; Foley et al., 2009a,b; Lawson and Wolfe, 2011; Moens et al., 2008; Sander et al., 2011a,b; Sood et al., 2006). To access these genes, we developed a Gateway plasmid-based system for germline specific mutagenesis based on previous work (Ablain et al., 2015; Kwan et al., 2007; Villefranc et al., 2007; Walhout et al., 2000). CRISPR/Cas9 mutagenesis is now a standard method in zebrafish and other organisms, and biallelic conversion events have been widely observed in mitotic cells (Ablain et al., 2015; Auer et al., 2014a,b; Barrangou, 2013; Blackburn et al., 2013; Hruscha et al., 2013; Hwang et al., 2013a,b). However, the effectiveness of CRISPR/Cas9 in meiotic cells, when there are four copies of each chromosome and distinct checkpoints and repair pathways, is unknown.

Briefly, we generated mutagenesis cassettes that include selectable markers [tissue-specific expression of fluorescent proteins (FPs)] and that express target guide RNAs ubiquitously (U6 promoter) and Cas9 from germline promoters [*bucky ball* (female meiotic cells) (Heim et al., 2014) and *ziwi* (all germ cells) (Leu and Draper, 2010); Fig. 1A,B, Fig. S1 and S2]. Using this approach, the germline is marked by GFP when the germline promoter is activated, and the cardiac myosin light chain promoter drives GFP in the heart to allow for earlier selection (Fig. 1A,C, Table S1). To generate transgenic animals, these cassettes, along with *transposase* RNA, were injected into embryos to generate stable lines by Tol2-mediated transgene integration (Kawakami, 2005, 2007; Kawakami et al., 2004, 1998). We anticipated mutations would be induced later by the *buc* mutagenesis cassette rather than using the *ziwi* mutagenesis cassette because the *buc* promoter is activated later in more advanced female germ cells compared with the *ziwi* promoter, which is expressed early in mitotic germ cells (Heim et al., 2014; Leu and Draper, 2010) (Fig. 2A). Hereafter, we refer to constructs and lines expressing *cas9* from the *buc* promoter as OMS for ovary mutagenesis system and those from the *ziwi* promoter as GMS for germline mutagenesis system. Because *buc* is activated only in females, founder males can be used to propagate the transgenes and to generate mutant alleles in subsequent generations. This will be valuable for mutations that cause female sterile phenotypes (oocytes arrest and no eggs are produced) or if the maternal-effect phenotypes are nonviable, e.g. *buc* mutants. Significantly, even if oocyte arrest occurs, histological assays can be used to examine affected gametes because the transgenic oocytes (OMS and GMS) or sperm (GMS) are marked with fluorescent reporters (Figs 1 and 2C-F). By sequencing the targeted region in marked oocytes or eggs, mutations induced in the germline can be identified.

Validation of OMS and GMS mutagenesis systems

Here, we report cassettes and recovered transgenic (Tg+) founders targeting two genes, *kif5ba* and *buc* (see Tables S1 and S2) both of which have known maternal-effect functions. Founders were identified based on GFP expression in their hearts and, in the case of females, transmission of GFP to their progeny. Although GFP should be a proxy for Cas9 because both proteins are produced from the same transcript (Fig. 1A), we confirmed that maternal Cas9 expression, like GFP, persists in embryos (Fig. 2B). Analysis of GFP-positive progeny of founders and F1 parents indicates that ubiquitous expression of guide RNAs is not toxic to germ cells (Fig. 2, Fig. S3). Similarly, germline Cas9 driven by *buc* (OMS) or *ziwi* (GMS) promoters is not toxic as fertile adults were recovered (Fig. 2C-E).

Phenocopy of *Mkif5ba* with mutagenesis vectors

Zebrafish kinesin I genes *kif5ba* and *kif5bb* function redundantly to promote craniofacial morphogenesis (Santos-Ledo et al., 2017). Double mutants lacking both fail to undergo proper jaw morphogenesis and are inviable (Santos-Ledo et al., 2017). Zygotic mutants disrupting *kif5ba* alone are viable; however, embryos lacking maternal *kif5ba* (*Mkif5ba*) fail to properly localize axis and germline determinants and consequently have dorsal-ventral patterning defects and lack primordial germ cells (PGCs) (Fig. 3A) (Campbell et al., 2015). As a proof of concept, we used the vector mutagenesis systems to disrupt *kif5ba*. We cloned a previously validated guide targeting the motor domain of *kif5ba* (Campbell et al., 2015) into the GMS cassette (hereafter called *Tg:GMS:kif5ba*) (see Tables S1 and S2).

Development (2021) 148, dev198010. doi:10.1242/dev.198010

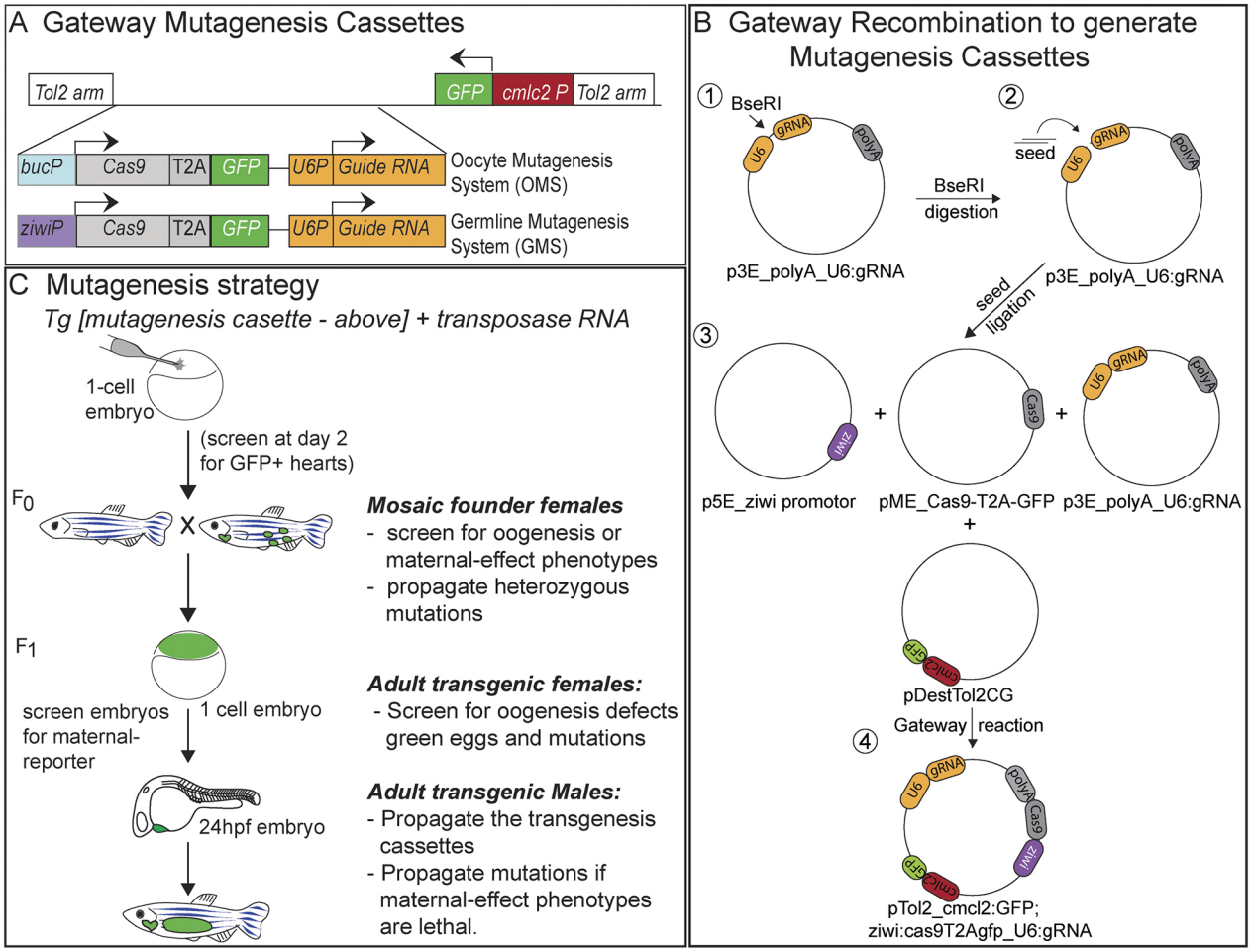


Fig. 1. Ovary and germ cell mutagenesis systems. (A) Schematic depicting the mutagenesis systems. Cas9 protein expressed from the *buc* (oocytes) or *ziwi* (all germ cells) promoter. Target-specific guides are expressed from the U6 promoter, and GFP is expressed from the *ziwi* promoter to mark the germline. Mutagenesis system vectors express Cas9 as a single transcript with GFP following a T2A cleavage signal. In addition, GFP is expressed from an independent heart promoter, *cardiac myosin light chain 2* (*cmlc2*), to permit earlier selection of transgenic animals. (B) Schematic of vectors and gateway-mediated recombination to generate OMS and GMS mutagenesis plasmids. (C) Schematic of mutagenesis strategy.

The restriction enzyme MboI cuts the wild-type allele; however, when mutated, MboI no longer cuts the mutant allele (Campbell et al., 2015). We used this assay to analyze the mutagenesis frequency in six GFP$^+$ F1 progeny of a *Tg:GMS:kif5ba* male founder and their F2 progeny (Fig. 3B, Fig. S3A,B). Analysis of genomic DNA from somatic tissue (fin) revealed that, as expected, if no germline mutations were induced, five of the six F1-progeny had homozygous wild-type somatic tissues (Fig. 3B). However, one female (female 3) was heterozygous, indicating *de novo* mutation of *kif5ba* occurred in her father's sperm (Fig. 3B). Next, we examined the progeny from pairwise intercrosses of *Tg:GMS:kif5ba* F1s to screen for germline mutations (Fig. 3B). Normally, a cross between two homozygous wild-type fish yields only homozygous wild-type progeny, whereas a cross between a homozygous wild-type fish and a heterozygote yields half homozygous wild-type and half heterozygous progeny. Instead, we found deviations from these expected genotypes in the progeny of *Tg:GMS:kif5ba* carriers, indicative of CRISPR/Cas9-mediated germline mutation (Fig. 3C). Sequencing of F2 progeny confirmed that new mutations were induced in the germline of *Tg:GMS:kif5ba* F1s. Both in frame and deleterious mutations were recovered (Fig. 3D).

Having confirmed induction of germline mutations, we examined the germline marker *nanos3* to determine whether germ cells were present in the GFP$^+$ progeny of *Tg:GMS:kif5ba* F1 females (Fig. 3, Fig. S4). As expected for mosaic loss of maternal *kif5ba* function, a fraction of the progeny from each *Tg:GMS:kif5ba* F1 female lacked germ cells expressing *nanos3* (Fig. 3E, Fig. S4). The penetrance of phenotypic embryos was nonmendelian and varied from female to female (ranging from 21-91%), with the highest frequency of phenotypic progeny from the *Tg:GMS:kif5ba* F1 mother that was already heterozygous for a mutation at the *kif5ba* locus. In addition, maternal *kif5ba* promotes dorso-ventral (DV) patterning by promoting the parallel vegetal microtubule array that mediates asymmetric distribution of dorsal factors (Campbell et al., 2015). To determine whether GMS-induced alleles recapitulated this *Mkif5ba* phenotype, we examined the phenotype of embryos from *Tg:GMS:kif5ba* F1 females at day 1 (d1). As expected, dorso-ventral phenotypes ranging from mild to severe dorsalization and axis duplication (V1 to V5 based on Kishimoto et al., 1997) were observed (Fig. 3F-J) among the green heart$^+$ progeny – the heart was not scoreable in embryos with duplicated axes (Fig. 3F, Fig. S3).

To confirm that mutations were induced in the germline and not the soma, we injected *kif5ba* guide RNA and Cas9 protein into *kif5bb*$^{e6/e6}$ homozygous mutants to determine whether the resulting somatic cell 'crispants' phenocopied the zygotic craniofacial defects observed in *kif5ba;kif5bb* double mutants at d5 (Santos-

Development (2021) 148, dev198010. doi:10.1242/dev.198010

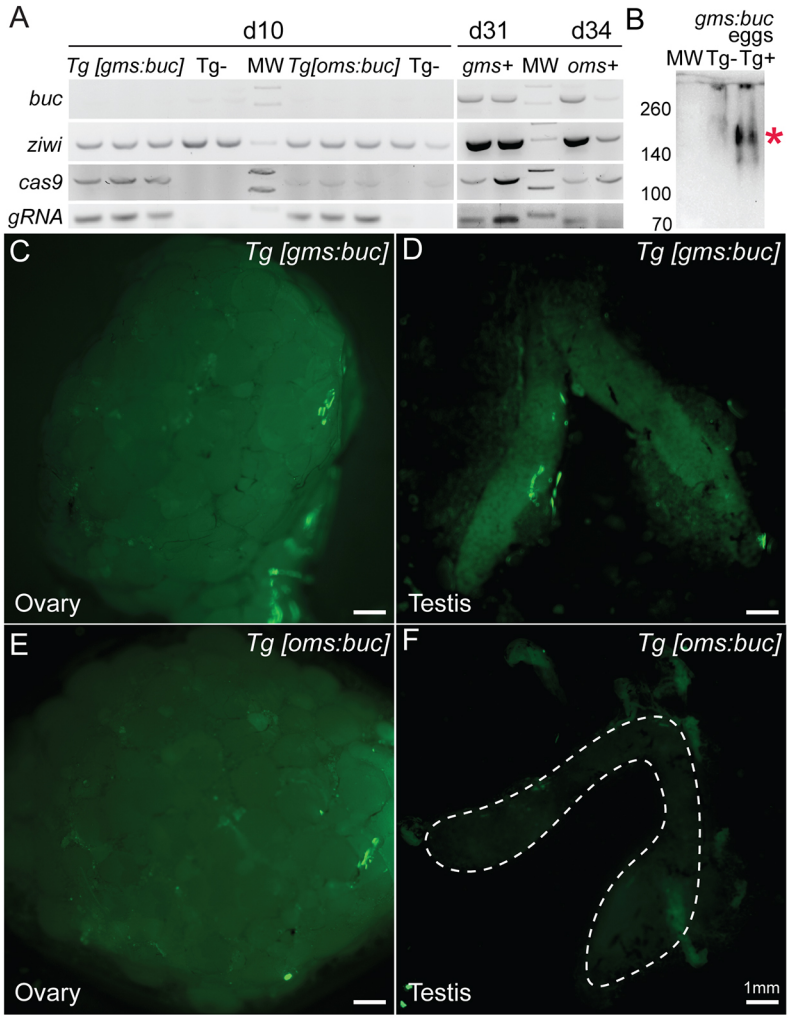


Fig. 2. Ubiquitous expression of Cas9 and guide RNAs are not toxic to germ cells or embryos. (A) RT-PCR of indicated genes. Expression from the *ziwi* promoter is detectable by d10 and *buc* is detectable later by d31. gRNAs are expressed in transgenic animals identified by GFP$^+$ hearts. *cas9* is expressed as expected for the respective promoters. (B) Western blot to detect maternal Cas9 protein (asterisk) in transgenic eggs from GMS*:buc* transgenic female (2 hpf; 10 egg equivalents/well). (C,D) Expression of GFP in ovary (C) and testis (D) of adult GMS transgenic animals. (E,F) Expression of GFP in ovary (E) but not testis (F) of adult OMS transgenic fish. Four ovaries and four testes were dissected and examined for each transgene. The testis is outlined

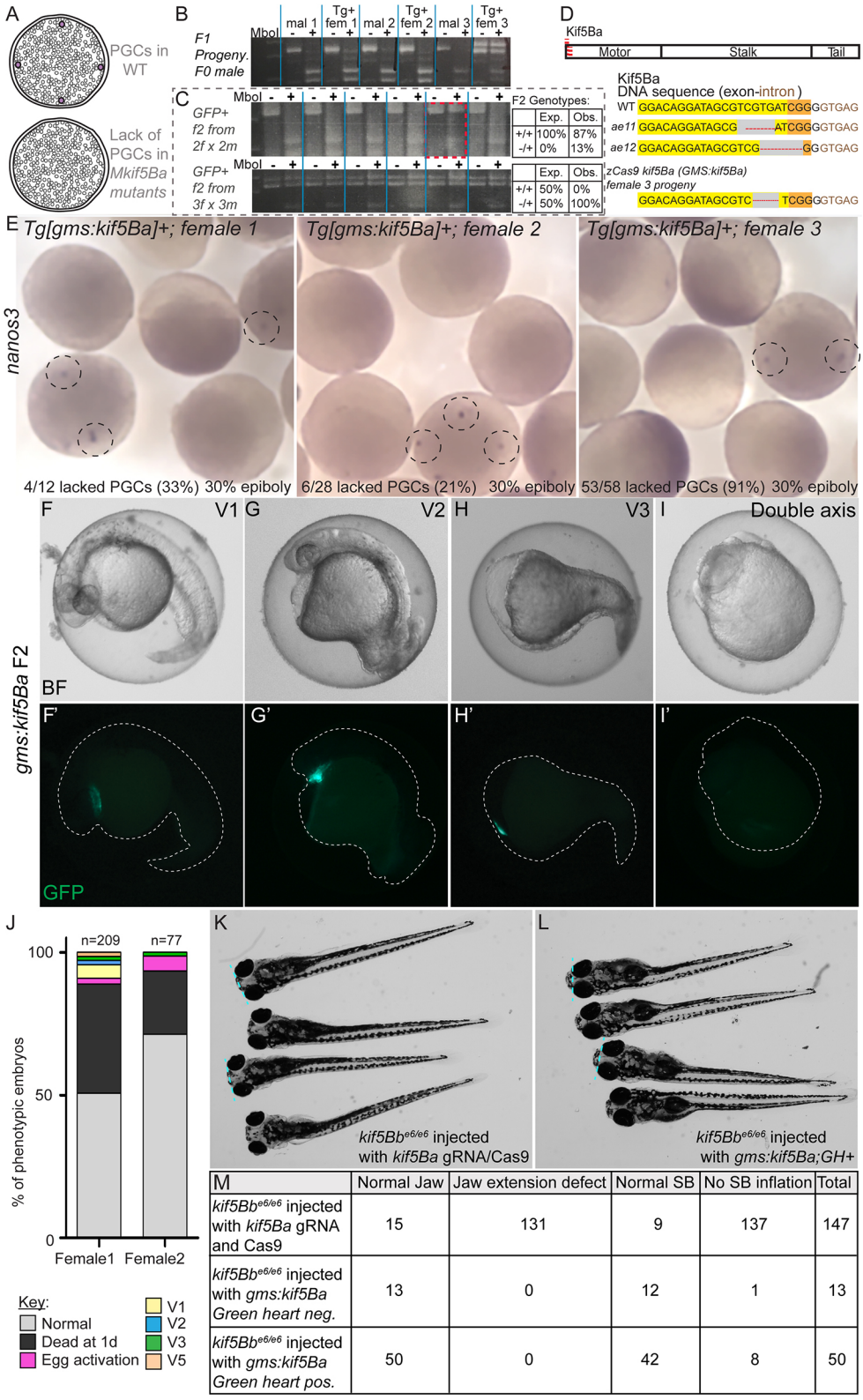
Ledo et al., 2017). As expected, jaw extension was compromised in 'crispants' (Fig. 3K,M). Next, we injected the *gms:kif5ba* plasmid system into *kif5bb* homozygous mutants to determine whether jaw defects were observed, which would be expected if there were leakage or somatic cell mutations. We observed no jaw defects in green heart-positive (transgenic$^+$) or in their transgene negative siblings on d5 (Fig. 3L,M). Moreover, the transgenic$^+$ animals were viable to adulthood, further indicating that somatic mutations were not induced because *kif5ba;kif5bb* double and compound mutant (mutant;heterozygote) fish are not viable (Santos-Ledo et al., 2017). Based on these results, we conclude that GMS-induced mutations in *kif5ba* can effectively phenocopy traditional maternal-effect loss of function *kif5ba* phenotypes, and that this system can be used to bypass somatic-lethal mutations.

Phenocopy of *buc*

Loss of *buc* results in failure to establish the animal-vegetal axis (Bontems et al., 2009; Dosch et al., 2004; Heim et al., 2014; Marlow and Mullins, 2008). To test the OMS and GMS systems at another locus, we generated guide RNAs targeting exon 4 of the *buc* gene and confirmed that the guides were mutagenic in transient assays (Fig. 4A, Tables S1 and S2). Mutagenic guides were cloned into the mutagenesis vectors and the resulting OMS*:buc* and OMS*:buc* plasmids were sequenced (Fig. 4A, Tables S1 and S2). We recovered one female and four male founders for OMS*:buc* (Fig. 4B,C, Fig. S3C). The single female OMS*:buc* founder produced embryos with either wild-type animal-vegetal polarity (n=22; 91.6%) or lacking polarity (n=2; 8.4%) (Fig. 4B,C, Fig. S3C). Thus, confirming the GMS system can be used to generate and assess maternal-effect phenotypes in just one generation, a significant advantage over traditional screens for maternal-effect functions in which phenotypes are detectable after four generations (Dosch et al., 2004; Wagner et al., 2004) and diploidized haploid screens (Pelegri et al., 2004; Pelegri and Mullins, 2004; Pelegri and Schulte-Merker, 1999).

To detect *de novo* mutations, T7 endonuclease assays were performed using genomic DNA from somatic tissues of the F1 progeny of two founder males. These analyses revealed 29% and 20% mutagenesis frequencies (Fig. 4D), indicating that expressing Cas9 from the *ziwi* promoter in the presence of guide RNA targeting *buc* induced germline mutations in founder males. Sequencing of genomic DNA confirmed the mutations detected by the T7 assays and revealed that all four F1 progeny of founder male E2 (three males and one female) carried the same mutation and that four female offspring from founder male E3 had a different mutant allele of *buc* (Fig. 4D, Fig. S5). Next, we examined the F1 females for *buc* phenotypes, specifically no animal-vegetal polarity and multiple micropyles – a somatic cell fate that is expanded in *buc* mutants (Heim et al., 2014; Marlow and Mullins, 2008). As expected for *buc* mutation, five F1 females produced progeny with *buc* phenotypes ranging in penetrance from 6-53% (Fig. 4E,I).

Development (2021) 148, dev198010. doi:10.1242/dev.198010



M	Normal Jaw	Jaw extension defect	Normal SB	No SB inflation	Total
kif5Bb$^{e6/e6}$ injected with *kif5Ba* gRNA and Cas9	15	131	9	137	147
kif5Bb$^{e6/e6}$ injected with *gms:kif5Ba Green heart neg.*	13	0	12	1	13
kif5Bb$^{e6/e6}$ injected with *gms:kif5Ba Green heart pos.*	50	0	42	8	50

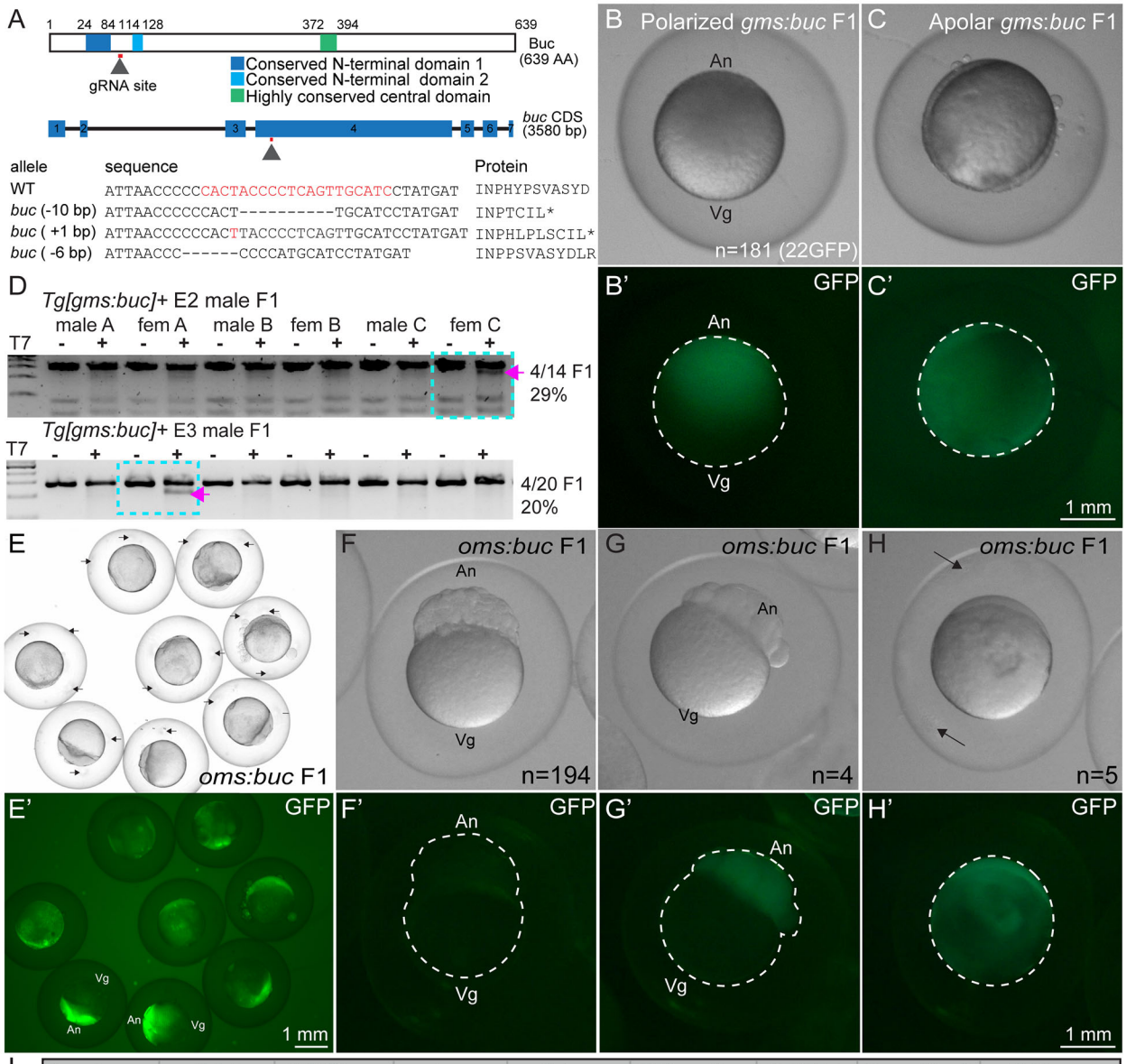
Fig. 3. Phenocopy of maternal *kif5ba* phenotypes. (A) Schematic of expected (and observed) wild-type and *Mkif5ba* PGC phenotypes. (B,C) Representative restriction enzyme-based assay for *kif5ba* mutations in (B) F1 and (C) F2 progeny of transgenic adults. Chart in C shows the expected and observed mutation frequencies for each cross. Plus indicates MboI digested; minus indicates undigested. #×# indicates the individuals intercrossed from F1 fish shown in B. (D) Schematic of Kif5Ba protein, the targeted region and representative mutant sequences. (E) *nanos3* staining of embryos from three independent clutches of F1 GMS:*kif5ba* mothers. Black dashed circles highlight PGCs, all others shown lack germ cells (see also Fig. S4). GMS:*kif5ba* F2. (F,F′) V1 ventralized embryo. (G,G′) V2 ventralized embryo. (H,H′) V3 ventralized embryo. (I,I′) V5 ventralized embryo. (J) Quantification of ventralization classes. (K) Representative *kif5bb*$^{e6/e6}$ larvae injected with *kif5ba* guide RNA and Cas9 (‘crispants’) at d5. (L) Representative *kif5bb*$^{e6/e6}$ larvae injected with GMS:*kif5ba* at d5. (K,L) Dashed blue lines are at the level of the eyes. Jaw extension defects are present in ‘crispants’ (K), whereas the jaws of GMS:*kif5ba* transgenic larvae are normal (L). (M) Quantification of jaw and swim bladder (SB) phenotypes.

Next, we generated stable transgenic lines for the OMS:*buc* alleles (Fig. S3). We recovered several male founders and one female founder whose progeny had wild-type animal-vegetal axes (Fig. 4F-G′) or lacked polarity (Fig. 4H,H′). As expected, the OMS system, which is expressed in meiotic cells when there are four copies of each chromosome that must be mutated, was less efficient than the GMS system, which is expressed earlier in mitotic germ cells which have only two copies of each chromosome.

Overall conclusions

Comparison of the OMS and GMS systems at two loci indicates that these systems can achieve disruption of gene function

Development (2021) 148, dev198010. doi:10.1242/dev.198010



I

Female (*gmsbuc*)	Total Offspring	Wild Type Offspring	*buc* Offspring	% *buc* offspring	GFP positive eggs	% GFP +	Green Heart Positive	% Green heart positive
A E2	291	264	27	9.3%	291	100%	264	91%
B E2	369	347	22	6%	366	99%	345	93%
C E2	243	207	36	14.8%	243	100%	214	88%
D E3	18	17	1	6%	18	100%	9	50
E E3	17	8	9	53%	17	100%	N/A nonviable	N/A

Fig. 4. Phenocopy of *buc* using GMS system. (A) Schematic depicting the site targeted in *buc* and representative mutant alleles. (B-C′) F1 progeny of F0 female transgenic for GMS:*buc* are (B,B′) polarized or (C,C′) lack polarity. (D) Representative T7 endonuclease assays to detect *buc* mutations. Mutagenesis frequencies for F1 progeny of two founder males. (E-H) Bright-field images and (E′-H′) maternal GFP expression in (E,E′) progeny of an OMS:*buc* F1 female and (F-H′) progeny of OMS:*buc* F0 female. (F,F′) GFP^{-} embryo with normal polarity. (G,G′) GFP^{+} embryo with normal polarity. (H,H′) GFP^{+} apolar embryo. (I) Table shows transmission frequency and phenotype penetrance of GMS:*buc* F1 progeny.

specifically in the germline at frequencies that match or exceed those of zygotic recessive alleles. The OMS system appears to be less efficient, possibly due to timing of expression (mitotic versus meiotic) and/or levels of expression from the promoters. Nonetheless, it may be suitable for genes transcribed in later oocytes, or for mutations that cause sterility using the GMS system. Detection of in-frame mutations indicates that phenotypic manifestation is an underrepresentation of mutagenesis efficiency, which encompasses both deleterious and non-deleterious mutations. Nondisruptive mutations are potentially limiting

Development (2021) 148, dev198010. doi:10.1242/dev.198010

because altered target sites cannot be further mutated. Here, only a single guide RNA was used; however, including multiple guides arrayed in tandem may yield large disruptive deletions. Although the frequency of phenotype detection varies for both GMS and OMS mutagenesis, this approach represents a significant advance in the tools available to study maternal-effect genes.

MATERIALS AND METHODS

Animals

Mutant fish strains were generated using Crispr-Cas9 mutagenesis with modifications (see the plasmids list in Table S1) to the plasmid backbone published previously (Ablain et al., 2015). All procedures and experimental protocols were performed in accordance with NIH guidelines and were approved by the Einstein (protocol #20140502) and Icahn School of Medicine at Mount Sinai Institutional (ISMMS) Animal Care and Use Committees (IACUC #2017-0114).

Primers

All primers are listed in Table S2.

OMS and GMS mutagenesis plasmids

OMS and GMS plasmids (Table S1) were created using the tissue-specific promoter system described by Ablain et al. (2015) (Fig. 1, see supplementary Materials and Methods). In brief, we digested a p3E_polyA_U6:gRNA (Fig. 1B; Ablain et al., 2015) using BseRI enzyme and then inserted annealed gene-specific gRNA targeting *kif5ba* and *buc*. For the gRNAs, previously validated gRNA sequences were used to target *kif5ba* (Campbell et al., 2015), and to target *buc,* new gRNAs were tested for mutagenic activity (Table S2). Gateway recombination reactions were then used to generate expression constructs with Cas9 driven by the *bucky ball* (Heim et al., 2014) or *ziwi* (Leu and Draper, 2010) promoter. Recombination order was confirmed by sequencing using the *ziwi* promoter or *buc* promoter, and the *cas9* and *U6* promoter primers (Table S2).

Stable transgenic lines

To generate stable OMS and GMS transgenic lines, Tol2 Transposase RNA was transcribed from pCS2FA-transposase (Kwan et al., 2007), and combined with OMS or GMS vector circular DNA (25 ng/µl each). Embryos were injected with 1 nl of the plasmid/transposase solution at the one-cell stage. Embryos with GFP-positive hearts were selected at day 2 (d2) and raised to generate founders.

Crispant experiments

The previously validated gRNAs targeting *kif5ba* (Campbell et al., 2015) and Cas9 or the *gms:kif5ba* mutagenesis vector (as described above) were injected into *kif5bb*$^{e6/e6}$ mutants. Larvae were scored for craniofacial morphology and swim bladder inflation at d5.

Mutation detection and genotyping

Genomic DNA was extracted from adult fins using standard procedures (Meeker et al., 2007). The genomic region surrounding the *kif5ba* target sequence was amplified using the primers 5′-GGAGTGCACCA-TTAAAGTCATGTG-3′ and 5′-GTCGGTGTCAAATATTGAGGTC-3′. The genomic region surrounding the *buc* target sequence was amplified using the primers 5′-TGCAGTATCCTGGCTATGTGAT-3′ and 5′-ACCA-CATCAGGGGTAGAAGAGA-3′ (Table S2). Products were then digested with T7 endonuclease and visualized on a gel to identify restriction patterns indicative of induced mutations.

Sequencing new alleles

Genomic DNA was extracted from adult fins using standard procedures (Meeker et al., 2007). The genomic region surrounding the *kif5ba* target sequence was amplified using the primers 5′-GGAGTGCACCA-TTAAAGTCATGTG-3′ and 5′-GTCGGTGTCAAATATTGAGGTC-3′. The genomic region surrounding the *buc* target sequence was amplified using the primers 5′-TGCAGTATCCTGGCTATGTGAT-3′ and 5′-ACCACATCAGGGGTAGAAGAGA-3′ (Table S2). After 35 cycles of PCR at 59°C and 57°C, for *kif5ba* and *buc*, respectively, PCR fragments were directly TA cloned into pCR4-TOPO vector (K457502, Invitrogen). After transformation, mini-prep DNA was prepared using Qiagen kits, and each plasmid was sequenced using universal primers on the vector. In this way, both the wild-type and any mutant alleles were detected.

Western blot

Fifty transgenic (identified by GFP expression) or non-transgenic (GFP negative) eggs were pooled together at 2 hpf. Samples were flash frozen and stored at −80°C. Samples were resuspended in 2×sample buffer with DTT at 1 µl/embryo or larvae. Samples were homogenized with a motorized pestle, centrifuged for 1 min, and incubated and boiled for 5 min prior to loading. 10 µl per sample was loaded in a 4-12% SDS-PAGE gel and proteins were transferred to PVDF membranes. Membranes were blocked in 5% milk in PBS for 1 h at room temperature. Anti-CRISPR-Cas9 antibody (Abcam, ab204448) was used at 1:1000 and incubated overnight. Membranes were washed for 3×5 min in TBS-Tween and then for 2×5 min in TBS. Rabbit-HRP secondary antibody was diluted 1:5000 and incubation was for 1 h at room temperature. Membranes were washed for 3×5 min in TBS-Tween and then for 2×5 min in TBS. Proteins were detected with ECL-Plus and chemiluminescence was imaged using a BioRad imager.

In situ hybridization

For *in situ* hybridization, embryos at the specified stages, were fixed in 4% paraformaldehyde overnight at 4°C. *In situ* hybridization was performed according to Thisse et al. (2004), except hybridization was performed at 65°C. In addition, maleic acid buffer [100 mM maleic acid (pH 8), 150 mM NaCl] was substituted for PBS during the antibody incubations, and BM Purple was used to visualize the RNA probes (Roche, 1442074).

Immunostaining and imaging

For whole-mount immunofluorescence stained shield, 30 hpf embryos or ovaries, tissues were fixed in 3.7% paraformaldehyde overnight at 4°C. The following day the samples were washed in PBS, dehydrated by washing in methanol, and then stored at −20°C. To visualize germ cells, chicken anti-GFP antibody (Invitrogen, A10262) was used at a 1:500 dilution. Secondary antibodies Alexafluor488 or Alexafluor-Cy3 (Molecular Probes) were diluted 1:500. Samples were mounted in Vectashield with DAPI and images were acquired using a Zeiss Axio Observer inverted microscope equipped with Apotome II and a CCD camera, a Zeiss Zoom dissecting scope equipped with Apotome II. Image processing was performed in Zenpro (Zeiss), ImageJ/FIJI, Adobe Photoshop and Adobe Illustrator.

RT-PCR

Eggs from transgenic mothers, day 10 (d10) trunks, and gonads dissected at d31-d34 from the specified transgenic genotypes were placed in RNA later and stored at −80°C until use. RNA later was removed and Trizol (Life Technologies) was added. RNA was extracted using the RNAeasy mini KIT (Qiagen) and the SuperScript IV VILO kit (Thermo Fisher) was used for cDNA preparation. RT-PCR was performed using the primers in Table S2. gRNAs were amplified using the target specific forward guide primer and the universal reverse primer. PCR products were resolved using a 1.5% Ultrapure agarose (Invitrogen) gel and visualized using a BioRad gel imager.

Acknowledgements

We thank members of the Marlow lab for helpful discussions, our animal care staff for fish care (Einstein and CCMS at ISMMS) and the Microscopy CoRE at Icahn School of Mount Sinai.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: O.K., F.L.M.; Methodology: S.B., O.K., F.L.M.; Validation: D.D., F.L.M.; Formal analysis: S.B., O.K., K.L., A.S.-L., F.L.M.; Investigation: S.B., O.K., K.L., A.S.-L., D.D., F.L.M.; Data curation: S.B., O.K., K.L., A.S.-L., D.D., F.L.M.;

Writing - original draft: F.L.M.; Writing - review & editing: S.B., O.K., K.L., A.S.-L., D.D., F.L.M.; Visualization: S.B., F.L.M.; Supervision: F.L.M.; Project administration: F.L.M.; Funding acquisition: F.L.M.

Funding

This work was supported by the National Institutes of Health (R21-HD091456 to F.L.M.). O.K. was supported by the National Institutes of Health (T32-GM007288) and by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (F30HD082903). Deposited in PMC for release after 12 months.

Peer review history

The peer review history is available online at https://journals.biologists.com/dev/article-lookup/doi/10.1242/dev.198010

References

Ablain, J., Durand, E. M., Yang, S., Zhou, Y. and Zon, L. I. (2015). A CRISPR/Cas9 vector system for tissue-specific gene disruption in zebrafish. *Dev. Cell* **32**, 756-764. doi:10.1016/j.devcel.2015.01.032

Abrams, E. W., Fuentes, R., Marlow, F. L., Kobayashi, M., Zhang, H., Lu, S., Kapp, L., Joseph, S. R., Kugath, A., Gupta, T., et al. (2020). Molecular genetics of maternally-controlled cell divisions. *PLoS Genet.* **16**, e1008652. doi:10.1371/journal.pgen.1008652

Ambartsumyan, G. and Clark, A. T. (2008). Aneuploidy and early human embryo development. *Hum. Mol. Genet.* **17**, R10-R15. doi:10.1093/hmg/ddn170

Auer, T. O., Duroure, K., Concordet, J. P. and Del Bene, F. (2014a). CRISPR/Cas9-mediated conversion of eGFP- into Gal4-transgenic lines in zebrafish. *Nat. Protoc.* **9**, 2823-2840. doi:10.1038/nprot.2014.187

Auer, T. O., Duroure, K., De Cian, A., Concordet, J. P. and Del Bene, F. (2014b). Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. *Genome Res.* **24**, 142-153. doi:10.1101/gr.161638.113

Barrangou, R. (2013). CRISPR-Cas systems and RNA-guided interference. *Wiley Interdiscip. Rev. RNA* **4**, 267-278. doi:10.1002/wrna.1159

Bennett, J. T., Stickney, H. L., Choi, W. Y., Ciruna, B., Talbot, W. S. and Schier, A. F. (2007). Maternal nodal and zebrafish embryogenesis. *Nature* **450**, E1-E2; discussion E2-4. doi:10.1038/nature06314

Bertho, S., Clapp, M., Banisch, T. U., Bandemer, J., Raz, E. and Marlow, F. L. (2021). Zebrafish dazl regulates cystogenesis and germline stem cell specification during the primordial germ cell to germline stem cell transition. *Development* **148**, dev187773. doi:10.1242/dev.187773

Blackburn, P. R., Campbell, J. M., Clark, K. J. and Ekker, S. C. (2013). The CRISPR system–keeping zebrafish gene targeting fresh. *Zebrafish* **10**, 116-118. doi:10.1089/zeb.2013.9999

Bontems, F., Stein, A., Marlow, F., Lyautey, J., Gupta, T., Mullins, M. C. and Dosch, R. (2009). Bucky ball organizes germ plasm assembly in zebrafish. *Curr. Biol.* **19**, 414-422. doi:10.1016/j.cub.2009.01.038

Borovina, A., Superina, S., Voskas, D. and Ciruna, B. (2010). Vangl2 directs the posterior tilting and asymmetric localization of motile primary cilia. *Nat. Cell Biol.* **12**, 407-412. doi:10.1038/ncb2042

Campbell, P. D., Heim, A. E., Smith, M. Z. and Marlow, F. L. (2015). Kinesin-1 interacts with Bucky ball to form germ cells and is required to pattern the zebrafish body axis. *Development* **142**, 2996-3008. doi:10.1242/dev.121434

Cao, Z., Mao, X. and Luo, L. (2019). Germline stem cells drive ovary regeneration in Zebrafish. *Cell Reports* **26**, 1709-1717.e1703. doi:10.1016/j.celrep.2019.01.061

Ciruna, B., Weidinger, G., Knaut, H., Thisse, B., Thisse, C., Raz, E. and Schier, A. F. (2002). Production of maternal-zygotic mutant zebrafish by germ-line replacement. *Proc. Natl. Acad. Sci. USA* **99**, 14919-14924. doi:10.1073/pnas.222459999

Ciruna, B., Jenny, A., Lee, D., Mlodzik, M. and Schier, A. F. (2006). Planar cell polarity signalling couples cell division and morphogenesis during neurulation. *Nature* **439**, 220-224. doi:10.1038/nature04375

Dosch, R., Wagner, D. S., Mintzer, K. A., Runke, G., Wiemelt, A. P. and Mullins, M. C. (2004). Maternal control of vertebrate development before the midblastula transition: mutants from the zebrafish I. *Dev. Cell* **6**, 771-780. doi:10.1016/j.devcel.2004.05.002

Doyon, Y., McCammon, J. M., Miller, J. C., Faraji, F., Ngo, C., Katibah, G. E., Amora, R., Hocking, T. D., Zhang, L., Rebar, E. J., et al. (2008). Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat. Biotechnol.* **26**, 702-708. doi:10.1038/nbt1409

Dranow, D. B., Tucker, R. P. and Draper, B. W. (2013). Germ cells are required to maintain a stable sexual phenotype in adult zebrafish. *Dev. Biol.* **376**, 43-50. doi:10.1016/j.ydbio.2013.01.016

Dranow, D. B., Hu, K., Bird, A. M., Lawry, S. T., Adams, M. T., Sanchez, A., Amatruda, J. F. and Draper, B. W. (2016). Bmp15 Is an Oocyte-Produced Signal Required for Maintenance of the Adult Female Sexual Phenotype in Zebrafish. *PLoS Genet.* **12**, e1006323. doi:10.1371/journal.pgen.1006323

Foley, J. E., Maeder, M. L., Pearlberg, J., Joung, J. K., Peterson, R. T. and Yeh, J. R. (2009a). Targeted mutagenesis in zebrafish using customized zinc-finger nucleases. *Nat. Protoc.* **4**, 1855-1867. doi:10.1038/nprot.2009.209

Foley, J. E., Yeh, J. R., Maeder, M. L., Reyon, D., Sander, J. D., Peterson, R. T. and Joung, J. K. (2009b). Rapid mutation of endogenous zebrafish genes using zinc finger nucleases made by Oligomerized Pool ENgineering (OPEN). *PLoS ONE* **4**, e4348. doi:10.1371/journal.pone.0004348

Hartung, O., Forbes, M. M. and Marlow, F. L. (2014). Zebrafish vasa is required for germ-cell differentiation and maintenance. *Mol. Reprod. Dev.* **81**, 946-961. doi:10.1002/mrd.22414

Hassold, T. and Hunt, P. (2007). Rescuing distal crossovers. *Nat. Genet.* **39**, 1187-1188. doi:10.1038/ng1007-1187

Hassold, T., Hall, H. and Hunt, P. (2007). The origin of human aneuploidy: where we have been, where we are going. *Hum. Mol. Genet.* **16**, R203-R208. doi:10.1093/hmg/ddm243

Heim, A. E., Hartung, O., Rothhamel, S., Ferreira, E., Jenny, A. and Marlow, F. L. (2014). Oocyte polarity requires a Bucky ball-dependent feedback amplification loop. *Development (Cambridge, England)* **141**, 842-854.

Hruscha, A., Krawitz, P., Rechenberg, A., Heinrich, V., Hecht, J., Haass, C. and Schmid, B. (2013). Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish. *Development* **140**, 4982-4987. doi:10.1242/dev.099085

Hwang, W. Y., Fu, Y., Reyon, D., Maeder, M. L., Kaini, P., Sander, J. D., Joung, J. K., Peterson, R. T. and Yeh, J. R. (2013a). Heritable and precise zebrafish genome editing using a CRISPR-Cas system. *PLoS ONE* **8**, e68708. doi:10.1371/journal.pone.0068708

Hwang, W. Y., Fu, Y., Reyon, D., Maeder, M. L., Tsai, S. Q., Sander, J. D., Peterson, R. T., Yeh, J. R. and Joung, J. K. (2013b). Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat. Biotechnol.* **31**, 227-229. doi:10.1038/nbt.2501

Kaufman, O. H., Lee, K., Martin, M., Rothhamel, S. and Marlow, F. L. (2018). rbpms2 functions in Balbiani body architecture and ovary fate. *PLoS Genet.* **14**, e1007489. doi:10.1371/journal.pgen.1007489

Kawakami, K. (2005). Transposon tools and methods in zebrafish. *Dev. Dyn.* **234**, 244-254. doi:10.1002/dvdy.20516

Kawakami, K. (2007). Tol2: a versatile gene transfer vector in vertebrates. *Genome Biol.* **8**(Suppl. 1), S7. doi:10.1186/gb-2007-8-s1-s7

Kawakami, K., Koga, A., Hori, H. and Shima, A. (1998). Excision of the tol2 transposable element of the medaka fish, Oryzias latipes, in zebrafish, Danio rerio. *Gene* **225**, 17-22. doi:10.1016/S0378-1119(98)00537-X

Kawakami, K., Imanaka, K., Itoh, M. and Taira, M. (2004). Excision of the Tol2 transposable element of the medaka fish Oryzias latipes in Xenopus laevis and Xenopus tropicalis. *Gene* **338**, 93-98. doi:10.1016/j.gene.2004.05.013

Kishimoto, Y., Lee, K. H., Zon, L., Hammerschmidt, M. and Schulte-Merker, S. (1997). The molecular nature of zebrafish swirl: BMP2 function is essential during early dorsoventral patterning. *Development* **124**, 4457-4466. doi:10.1242/dev.124.22.4457

Kwan, K. M., Fujimoto, E., Grabher, C., Mangum, B. D., Hardy, M. E., Campbell, D. S., Parant, J. M., Yost, H. J., Kanki, J. P. and Chien, C. B. (2007). The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Dev. Dyn.* **236**, 3088-3099. doi:10.1002/dvdy.21343

Lawson, N. D. and Wolfe, S. A. (2011). Forward and reverse genetic approaches for the analysis of vertebrate development in the zebrafish. *Dev. Cell* **21**, 48-64. doi:10.1016/j.devcel.2011.06.007

Leu, D. H. and Draper, B. W. (2010). The ziwi promoter drives germline-specific gene expression in zebrafish. *Dev. Dyn.* **239**, 2714-2721. doi:10.1002/dvdy.22404

Marlow, F. L. (2010). *Maternal Control of Development in Vertebrates: My Mother Made Me Do It!*. San Rafael, CA.

Marlow, F. L. and Mullins, M. C. (2008). Bucky ball functions in Balbiani body assembly and animal-vegetal polarity in the oocyte and follicle cell layer in zebrafish. *Dev. Biol.* **321**, 40-50. doi:10.1016/j.ydbio.2008.05.557

Meeker, N. D., Hutchinson, S. A., Ho, L. and Trede, N. S. (2007). Method for isolation of PCR-ready genomic DNA from zebrafish tissues. *BioTechniques* **43**, 612. 614.

Moens, C. B., Donn, T. M., Wolf-Saxon, E. R. and Ma, T. P. (2008). Reverse genetics in zebrafish by TILLING. *Brief. Funct. Genomic. Proteomic.* **7**, 454-459. doi:10.1093/bfgp/eln046

Pelegri, F. and Mullins, M. C. (2004). Genetic screens for maternal-effect mutations. *Methods Cell Biol.* **77**, 21-51. doi:10.1016/S0091-679X(04)77002-8

Pelegri, F. and Schulte-Merker, S. (1999). A gynogenesis-based screen for maternal-effect genes in the zebrafish, Danio rerio. *Methods Cell Biol.* **60**, 1-20.

Pelegri, F., Dekens, M. P., Schulte-Merker, S., Maischein, H. M., Weiler, C. and Nusslein-Volhard, C. (2004). Identification of recessive maternal-effect mutations in the zebrafish using a gynogenesis-based method. *Dev. Dyn.* **231**, 324-335. doi:10.1002/dvdy.20145

Rodriguez-Mari, A. and Postlethwait, J. H. (2011). The role of Fanconi anemia/BRCA genes in zebrafish sex determination. *Methods Cell Biol.* **105**, 461-490. doi:10.1016/B978-0-12-381320-6.00020-5

Rodriguez-Mari, A., Canestro, C., Bremiller, R. A., Nguyen-Johnson, A., Asakawa, K., Kawakami, K. and Postlethwait, J. H. (2010). Sex reversal in zebrafish fancl mutants is caused by Tp53-mediated germ cell apoptosis. *PLoS Genet.* **6**, e1001034. doi:10.1371/journal.pgen.1001034

Rodriguez-Mari, A., Wilson, C., Titus, T. A., Canestro, C., BreMiller, R. A., Yan, Y. L., Nanda, I., Johnston, A., Kanki, J. P., Gray, E. M., et al. (2011). Roles of brca2 (fancd1) in oocyte nuclear architecture, gametogenesis, gonad tumors, and genome stability in zebrafish. *PLoS Genet.* **7**, e1001357. doi:10.1371/journal.pgen.1001357

Romano, S., Kaufman, O. H. and Marlow, F. L. (2020). Loss of dmrt1 restores zebrafish female fates in the absence of cyp19a1a but not rbpms2a/b. *Development* **147**, dev190942. doi:10.1242/dev.190942

Sander, J. D., Cade, L., Khayter, C., Reyon, D., Peterson, R. T., Joung, J. K. and Yeh, J. R. (2011a). Targeted gene disruption in somatic zebrafish cells using engineered TALENs. *Nat. Biotechnol.* **29**, 697-698. doi:10.1038/nbt.1934

Sander, J. D., Yeh, J. R., Peterson, R. T. and Joung, J. K. (2011b). Engineering zinc finger nucleases for targeted mutagenesis of zebrafish. *Methods Cell Biol.* **104**, 51-58. doi:10.1016/B978-0-12-374814-0.00003-3

Santos-Ledo, A., Garcia-Macia, M., Campbell, P. D., Gronska, M. and Marlow, F. L. (2017). Kinesin-1 promotes chondrocyte maintenance during skeletal morphogenesis. *PLoS Genet.* **13**, e1006918. doi:10.1371/journal.pgen.1006918

Sato, Y., Hilbert, L., Oda, H., Wan, Y., Heddleston, J. M., Chew, T. L., Zaburdaev, V., Keller, P., Lionnet, T., Vastenhouw, N., et al. (2019). Histone H3K27 acetylation precedes active transcription during zebrafish zygotic genome activation as revealed by live-cell analysis. *Development* **146**, dev179127. doi:10.1242/dev.179127

Siegfried, K. R. and Nusslein-Volhard, C. (2008). Germ line control of female sex determination in zebrafish. *Dev. Biol.* **324**, 277-287. doi:10.1016/j.ydbio.2008.09.025

Slanchev, K., Stebler, J., de la Cueva-Mendez, G. and Raz, E. (2005). Development without germ cells: the role of the germ line in zebrafish sex differentiation. *Proc. Natl. Acad. Sci. USA* **102**, 4074-4079. doi:10.1073/pnas.0407475102

Sood, R., English, M. A., Jones, M., Mullikin, J., Wang, D. M., Anderson, M., Wu, D., Chandrasekharappa, S. C., Yu, J., Zhang, J., et al. (2006). Methods for reverse genetic screening in zebrafish by resequencing and TILLING. *Methods* **39**, 220-227. doi:10.1016/j.ymeth.2006.04.012

Thisse, B., Heyer, V., Lux, A., Alunni, V., Degrave, A., Seiliez, I., Kirchner, J., Parkhill, J. P. and Thisse, C. (2004). Spatial and temporal expression of the zebrafish genome by large-scale in situ hybridization screening. *Methods Cell Biol.* **77**, 505-519. doi:10.1016/S0091-679X(04)77027-2

Vastenhouw, N. L., Cao, W. X. and Lipshitz, H. D. (2019). The maternal-to-zygotic transition revisited. *Development* **146**, dev161471. doi:10.1242/dev.161471

Villefranc, J. A., Amigo, J. and Lawson, N. D. (2007). Gateway compatible vectors for analysis of gene function in the zebrafish. *Dev. Dyn.* **236**, 3077-3087. doi:10.1002/dvdy.21354

Wagner, D. S., Dosch, R., Mintzer, K. A., Wiemelt, A. P. and Mullins, M. C. (2004). Maternal control of development at the midblastula transition and beyond: mutants from the zebrafish II. *Dev. Cell* **6**, 781-790. doi:10.1016/j.devcel.2004.04.001

Walhout, A. J., Temple, G. F., Brasch, M. A., Hartley, J. L., Lorson, M. A., van den Heuvel, S. and Vidal, M. (2000). GATEWAY recombinational cloning: application to the cloning of large numbers of open reading frames or ORFeomes. *Methods Enzymol.* **328**, 575-592. doi:10.1016/S0076-6879(00)28419-X

Weidinger, G., Stebler, J., Slanchev, K., Dumstrei, K., Wise, C., Lovell-Badge, R., Thisse, C., Thisse, B. and Raz, E. (2003). . dead end, a novel vertebrate germ plasm component, is required for zebrafish primordial germ cell migration and survival. *Curr. Biol.* **13**, 1429-1434. doi:10.1016/S0960-9822(03)00537-2

Williams, M. L. K., Sawada, A., Budine, T., Yin, C., Gontarz, P. and Solnica-Krezel, L. (2018). Gon4l regulates notochord boundary formation and cell polarity underlying axis extension by repressing adhesion genes. *Nat. Commun.* **9**, 1319. doi:10.1038/s41467-018-03715-w

Wu, D. and Dean, J. (2020). Maternal factors regulating preimplantation development in mice. *Curr. Top. Dev. Biol.* **140**, 317-340. doi:10.1016/bs.ctdb.2019.10.006

Wu, E. and Vastenhouw, N. L. (2020). From mother to embryo: a molecular perspective on zygotic genome activation. *Curr. Top. Dev. Biol.* **140**, 209-254. doi:10.1016/bs.ctdb.2020.02.002

Wu, K., Song, W., Zhang, Z. and Ge, W. (2020). Disruption of dmrt1 rescues the all-male phenotype of the cyp19a1a mutant in zebrafish – a novel insight into the roles of aromatase/estrogens in gonadal differentiation and early folliculogenesis. *Development* **147**, dev182758. doi:10.1242/dev.182758

Zhao, L. Y., Niu, Y., Santiago, A., Liu, J., Albert, S. H., Robertson, K. D. and Liao, D. (2006). An EBF3-mediated transcriptional program that induces cell cycle arrest and apoptosis. *Cancer Res.* **66**, 9445-9452. doi:10.1158/0008-5472.CAN-06-1713

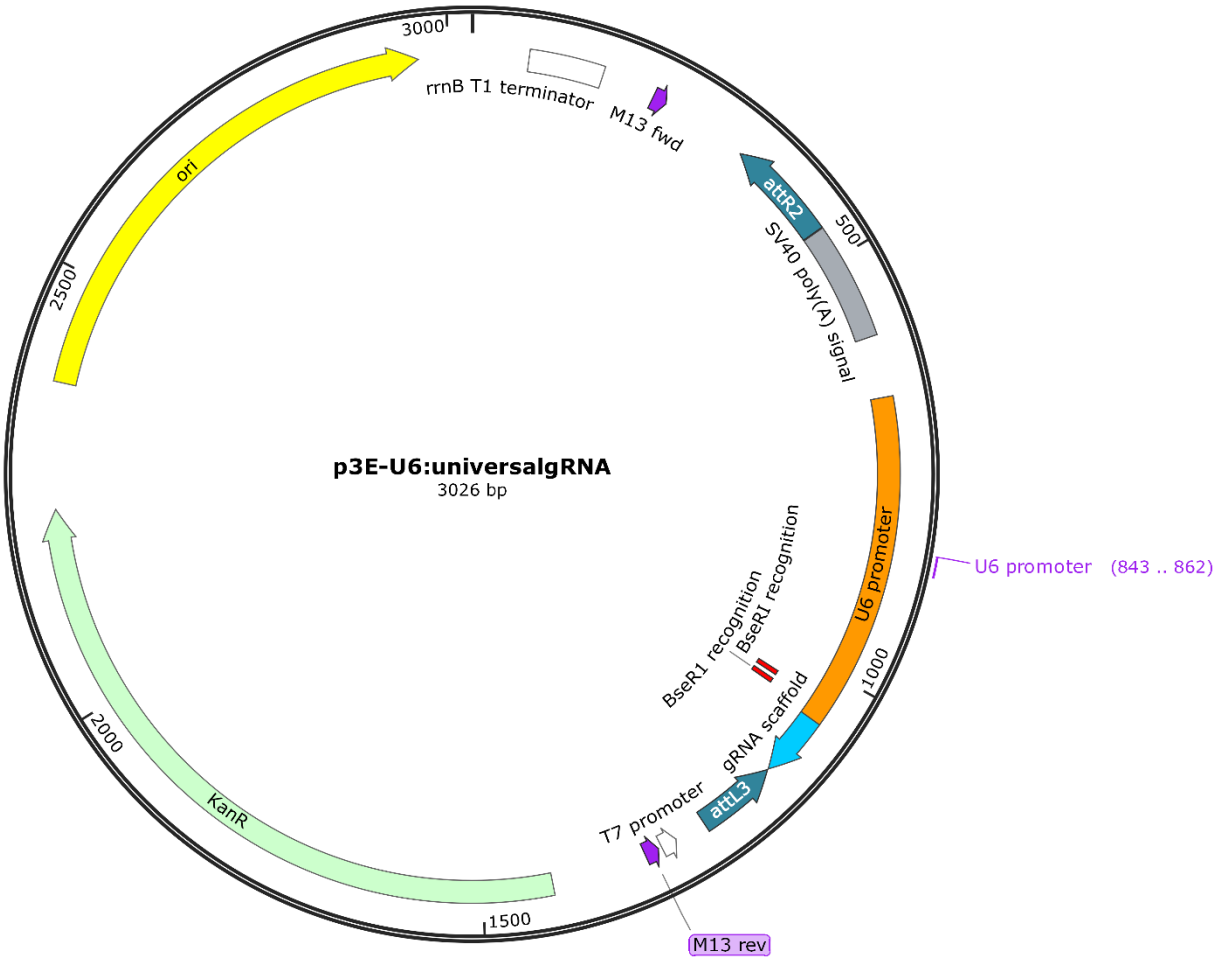


Figure S1. Plasmid map of p3E_polyA_U6:universalgRNA. The plasmid contains attR2 and attL3 recombination sites (dark turquoise), SV40 poly(A) signal (grey), U6 promoter (orange), BseR1 recognition site (red) where the specific gRNA will be placed, the gRNA scaffold (light blue), and the sequencing primer (Forward direction).

TGGCGGGCGTCCTGCCCGCCACCCTCCGGGCCGTTGCTTCACAACGTTCAAATCCGCTCCCGGCG

GATTTGTCCTACTCAGGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTCCGA
CTGAGCCTTTCGTTTTATTTGATGCCTGGCAGTTCCCTACTCTCGCGTTAACGCTAGCATGGATGTTT
TCCCAGTCACGACGTTGTAAAACGACGGCCAGTCTTAAGCTCGGGCCCTGCAGCTCTAGAGCTCGA
ATTCTACAGGTCACTAATACCATCTAAGTAGTTGGTTTACAGGTCACTAATACCATCTAAGTAGTTGGT
TCATAGTGACTGCATATGTTGTGTTTTACAGTATTATGTAGTCTGTTTTTTATGCAAAATCTAATTTAAT
ATATTGATATTTATATCATTTTACGTTTCTCGTTCAACTTTCTTGTACAAAGTGGGGGATCCAGACATG
ATAAGATACATTGATGAGTTTGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGA
AATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATTG
CATTCATTTTATGTTTCAGGTTCAGGGGGAGGTGTGGGAGGTTTTTTCATCATCGATGCGTCTTTTGT
TCTGGTCATCAAGGAGGGGGGAATGTTCTGCGCATGCCTGTGGGGGAGGGAGAAGGACACGTCAC
TGAAAACGTCCCTGCATCACACCGAGACACCCAATCACTCAAGCCGAGACCAGATAATTTTGCATAT
GCTTTACAGTTTGAAAAATACCACGGTAAACCCTCACACAAACTCTGGATTTGAGATCTTTCAGGTTT
TATCAGTTTGCAGGTTTATGTCACCATGATATAGGGTCAGACTTGATCTAAGGGAGCTGAATAAGTGG
TTTAGTCACTCACCACCTCCCAAAAACATACCCAGAAGTCCCTGGTATATATAGCTCTCCCTCCAGCT
CTTGGTTCGGCTAGCACTCCTCAGTGACGAGGAGTCAGTAGCGTTTTAGAGCTAGAAATAGCAAGTT
AAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTCAACTTTATTATACAA
AGTTGGCATTATAAAAAAGCATTGCTTATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAA
AATCATTATTTGGAGCTCCATGGTAGCGTTAACGCGGCCGCGATATCCCCTATAGTGAGTCGTATTA
CATGGTCATAGCTGTTTCCTGGCAGCTCTGGCCCGTGTCTCAAAATCTCTGATGTTACATTGCACAA
GATAAAAATATATCATCATGAACAATAAAACTGTCTGCTTACATAAACAGTAATACAAGGGGTGTTATG
AGCCATATTCAACGGGAAACGTCGAGGCCGCGATTAAATTCCAACATGGATGCTGATTTATATGGGT
ATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGCTTGTATGGGAAGCCCG
ATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGT
CAGACTAAACTGGCTGACGGAATTTATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATG
ATGCATGGTTACTCACCACTGCGATCCCCGGAAAAACAGCATTCCAGGTATTAGAAGAATATCCTGA
TTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGATTCCTGTTTGTA
ATTGTCCTTTTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAATAACGGTTTG
GTTGATGCGAGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGC
ATAAACTTTTGCCATTCTCACCGGATTCAGTCGTCACTCATGGTGATTTCTCACTTGATAACCTTATTT
TTGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGACGAGTCGGAATCGCAGACCGATACCAGGA

TCTTGCCATCCTATGGAACTGCCTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAAT
ATGGTATTGATAATCCTGATATGAATAAATTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAATCAG
AATTGGTTAATTGGTTGTAACACTGGCAGAGCATTACGCTGACTTGACGGGACGGCGCAAGCTCATG
ACCAAAATCCCTTAACGTGAGTTACGCGTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAA
GGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACC
AGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGA
GCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAG
CACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTG
TCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGG
GTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGC
ATTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTC
GGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGG
GTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAA
AAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTT

Figure S2. P3E_polyA_U6:universalgRNA sequence.
attR2 and attL3 (green), SV 40 polyA (grey), U6 promoter (orange), primer (purple), BseR1 enzyme site (red), gRNA scaffold (blue).

A F0 transmission frequencies

Founders	Transmission freq - GH positive
Tg[pGH:Ziwi:cas9T2AGFP_U6kif5Ba gRNA] 3073-1 male	0.27
Tg[pGH:Ziwi:cas9T2AGFP_U6kif5Ba gRNA] 3590-1 male	0.01
Tg[pGH:Ziwi:cas9T2AGFP_U6kif5Ba gRNA] 3590-5-20 male	0.03

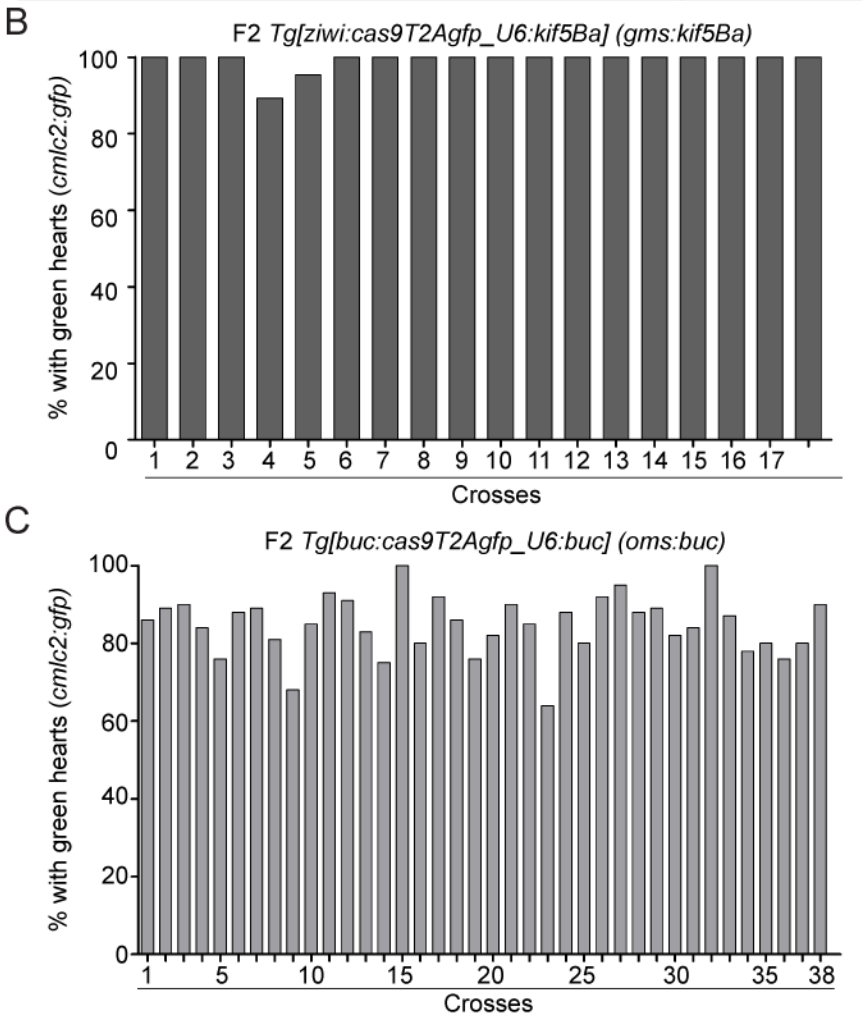


Figure S3. Germline transgene transmission. A) Representative F0 transmission frequencies (GH positive indicates - GFP positive hearts). B) Quantification of positive green heart expression among *Tg[buc:cas9T2Agfp_U6:kif5B]* (*gms:kif5B)* embryos (n=18). C) Quantification of positive green heart expression among *Tg[ziwi:cas9T2Agfp_U6:buc] (gms:buc)* embryos on d3 (n=38).

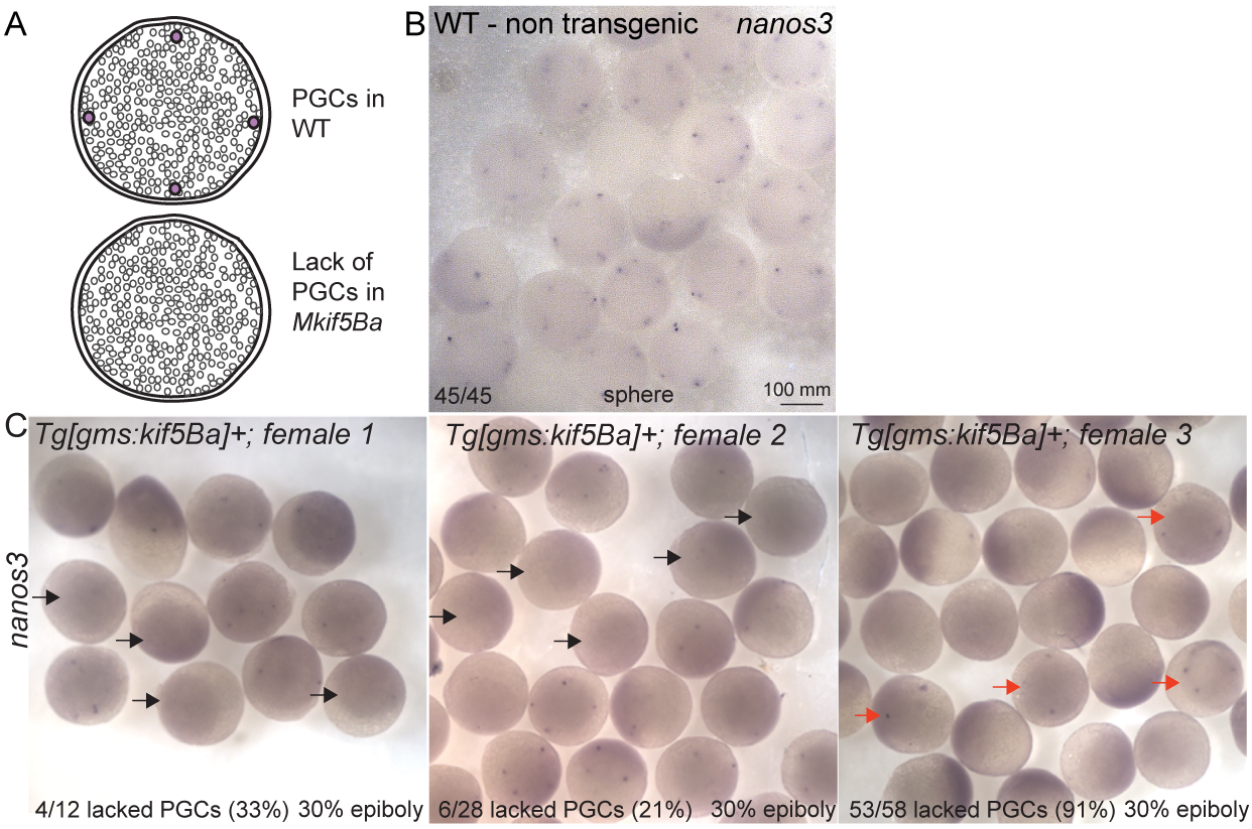


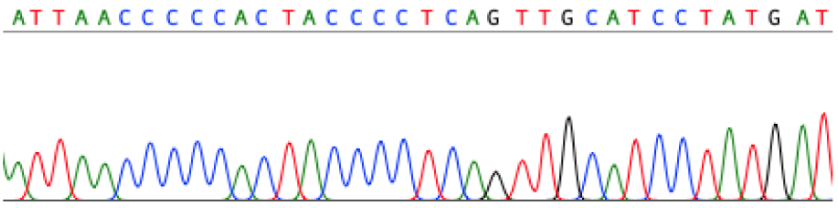
Figure S4. Phenocopy of maternal *kif5Ba* PGC phenotype. A) Schematic of expected wild-type and *Mkif5Ba* PGC phenotypes. B,C) *nanos3* staining in (B) non-transgenic control and (C) three independent clutches of F1 *gms:kif5Ba* transgenic mothers – the panels correspond to those in main Figure 3E. Black arrows indicate progeny lacking germ cells and red arrows indicate the few embryos with germ cells in female 3.

A

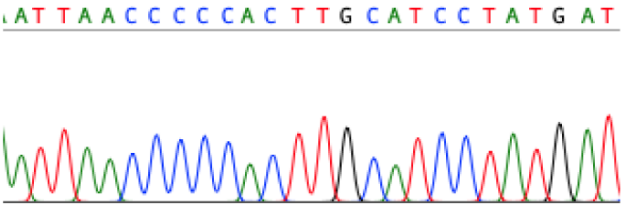
allele	sequence	Protein
WT	ATTAACCCCCCACTACCCCTCAGTTGCATCCTATGAT	INPHYPSVASYD
buc (-10 bp)	ATTAACCCCCCACT----------TGCATCCTATGAT	INPTCIL*
buc (+1 bp)	ATTAACCCCCCACTTACCCCTCAGTTGCATCCTATGAT	INPHLPLSCIL*
buc (-6 bp)	ATTAACCC------CCCCATGCATCCTATGAT	INPPSVASYDLR

B

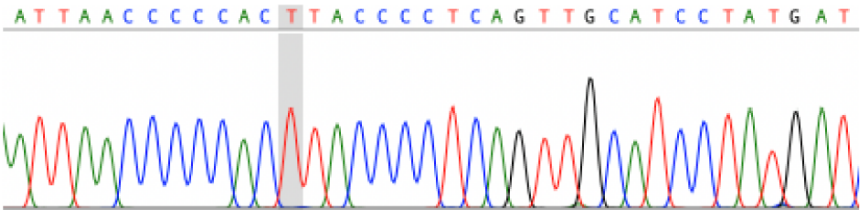
WT - Chromatogram



buc (- 10 bp) - Chromatogram



buc (+1 bp) - Chromatogram



buc (- 6 bp) - Chromatogram

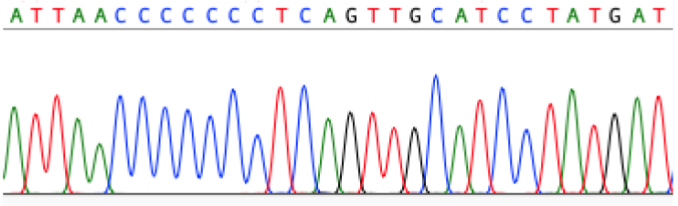


Figure S5. Sequence and chromatogram from recovered *buc* germline mutations from *gms:buc* mothers. (A) Sequence of the region flanking the *buc* gRNA site and recovered sequences after Cas9 cleavage. (B) Chromatogram of the WT sequence and the mutated sequences in eggs from gms:buc mothers.

Table S1. Plasmids generated/used in this study

Marlow Lab Database Number	Plasmid Name	Other Info.
#60	*p5E pBD119 ziwi promoter*	(Leu and Draper, 2010)
#190	*2K Buc promoter in PDONR (p5E-Buc)*	(Heim et al., 2014)
#878	*Tol2-R4/R3 cmcl2:gfp*	Tol2 sites surrounding R4/R3 att sites, with cmcl2:gfp transgenesis marker, Tol2 v1.0 (Kwan et al., 2007)
#1190	*pME-Cas9*	Addgene 63154 (Zon lab) (Ablain et al., 2015)
#1191	*pME-cas9-T2A-GFP*	Addgene 63155 (Zon lab) (Ablain et al., 2015)
#1195	*p3E_pA_U6:kif5Ba_gRNA*	Guide from (Campbell et al., 2015)
#1244	*pGH ziwi:cas9-T2A-GFP u6:kif5Ba gRNA (GMS)*	Guide from (Campbell et al., 2015)
#1245	*pGH buc:cas9-T2A-GFP u6:kif5Ba gRNA (OMS)*	Guide from (Campbell et al., 2015)
#1246	*p3E_pA_u6:universal gRNA*	
#1268	*pGHziwiCas9T2AGFPU6bucgRNA*	
#1269	*pGHbucCas9T2AGFPU6bucgRNA*	
#1272	*p3E-pA-U6bucgRNA*	

Table S2

Click here to download Table S2

Supplementary materials and methods

Protocol for germline-specific gene disruption in zebrafish

Adapted from (Ablain et al., 2015)

Overview

This protocol is based on (Ablain et al., 2015) tissue-specific gene disruption protocol in zebrafish. The method allows gene inactivation in zebrafish in a germline-specific manner. It can be used to analyze maternal effect genes in F1 embryos and generate stable tissue-specific knock-out lines to analyze maternal effect genes. It takes advantage of the Tol2 transposase technology to integrate in the fish genome a vector expressing a guide RNA (gRNA) from a ubiquitous zebrafish U6 promoter and Cas9 under the control of a tissue-specific promoter. This protocol comprises 5 steps: 1) the identification of efficient CRISPR target sequences in the gene of interest; 2) the annealing of gene-specific oligonucleotides; 3) the construction of the germline-specific CRISPR vector; 4)

the injection of the CRISPR construct in zebrafish embryos; 5) the phenotypic analysis and generation of stable lines (Fig. P1).

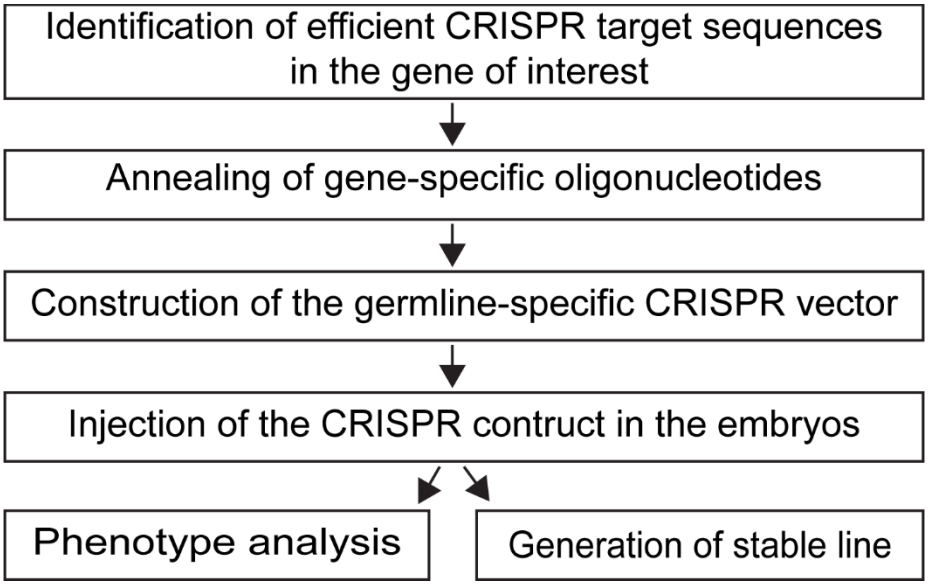


Figure P1 – Workflow of germline-specific gene disruption

Reagents

- Destination vector for Gateway (e.g. pDestTol2CG2)
- Middle entry vector for Gateway containing Cas9 (e.g. pME-Cas9 or pME-Cas9-T2A-GFP, available through Addgene)
- 5' entry vector for Gateway containing the germline-specific promoter of interest (p5E_*ziwi* promoter or p5E_*buc* promoter)
- 3' entry vector for Gateway containing a polyA sequence (p3E_polyA_U6:universalgRNA)
- Gene-specific oligonucleotides (see below)
- BseRI enzyme (New England Biolabs)
- Gateway LR clonase II (Invitrogen)
- T4 DNA ligase (New England Biolabs)
- Gel extraction kit or PCR-purification kit (Qiagen)

Procedure

All the steps except step 3 are identical to Ablain et al procedure. (Ablain et al., 2015)

1. Identification of efficient CRISPR target sequences in the gene(s) of interest

Pick several (3 to 6) CRISPR target sequences in each gene of interest using available tools (Hsu et al., 2013; Montague et al., 2014). Produce the gRNAs *in vitro* as per usual procedures (Gagnon et al., 2014b; Hwang et al., 2013). Inject the gRNAs along with Cas9 protein or mRNA into one-cell stage embryos of a WT strain. Extract DNA from injected embryos at 24 or 48 hpf by the HotSHOT method (Meeker et al., 2007) and assess mutation rates at target loci by sequencing or enzymatic assays (e.g. T7E1 assay, Surveyor assay) (Gagnon et al., 2014b; Kim et al., 2009). Proceed with the target sequences that have shown effective targeting (we usually only keep target sequences for which the mutation rates exceed 10%).

Note: *alternatively, it is possible to test the efficiency of target sequences directly in the context of the Tol2 vector by cloning various target sequences in the U6:gRNA cassette of a vector expressing Cas9 under the control of a ubiquitous promoter.*

2. Annealing of gene-specific oligonucleotides

Order 22-mer, unmodified oligonucleotides as follows:
Forward: target sequence (20 bases)-GT
Reverse: reverse complement of target sequence-GA
Note: *the target sequence must start with a G. If not, replace the first base by a G. This will introduce a mismatch but most mismatches at the 5' end of the target sequence are well tolerated.*

Example: for CRISPR target sequence GGTGGGAGAGTGGATGGCTG, order GGTGGGAGAGTGGATGGCTGGT (forward) and CAGCCATCCACTCTCCCACCGA (reverse).
Anneal the two oligos in thermocycler (5 min. at 95°C, -1°C/min. down to 20°C).

3. Construction of the germline-specific CRISPR vector

Clone the gene-specific seed sequence into the p3E_polyA_U6:universal gRNA predigested with BseRI and then perform the Gateway reaction with the germline-specific promoter (ziwi or buc) and Cas9 (Fig. 1B).

- Digest the p3E_polyA_U6:universalgRNA with BseRI enzyme.
- Purify on 1% agarose gel or column.
- Ligate the gene-specific seed sequence into BseRI-digested p3E_polyA_U6:universalgRNA vector (1:20 vector:insert molar ratio).
- Transform chemically competent bacteria, plate on LB Agar and select with Kanamycin

- Extract plasmid DNA from 2-3 distinct colonies and sequence using the following primer: CCTCACACAAACTCTGGATT to check the insertion of the specific gRNA.

- Perform the Gateway reaction (Hartley et al., 2000) with the destination vector, a 5' entry vector containing a tissue-specific promoter of interest, a middle entry vector containing zebrafish codon-optimized Cas9 and p3E-polyA, according to manufacturer's protocol.

- Transform chemically competent bacteria, plate on LB Agar and select with ampicillin.

- Extract plasmid DNA from 2-3 colonies and check correct recombination by digestion or sequencing (*ziwi* or *buc*, *Cas9*, *U6* promoter primer listed in the table).

4. Injection of the CRISPR construct in zebrafish embryos

Mix and inject 20-30 pg of CRISPR vector and 20 pg of Tol2 mRNA into one-cell stage embryos (Kawakami et al., 2004). For reliable phenotypic analyses, we recommend injecting >50 embryos per construct. Vectors expressing gRNAs targeting an irrelevant gene or driving Cas9 expression in a different tissue can be used as negative controls. Allow injected embryos to develop at 28.5°C.

5. Phenotypic analysis and generation of stable lines

Sort injected F0 embryos based on the expression of a transgenesis marker present either in the destination vector (e.g. cmlc2:GFP) or in the middle entry part of the vector (e.g. T2A-GFP). Only positive embryos should be considered for further analysis.
For the generation of stable lines, raise positive F0 fish to adulthood. Back-cross them to the strain used for injection and sort positive F1 embryos (according to the expression of the transgenesis marker). F1 embryos can be analyzed phenotypically or raised to adulthood.
Note: *depending on the transgenesis marker used, it may be possible to evaluate the level of mosaicism in injected embryos. In that case, injected embryos could be further sorted according to their level of mosaicism.*

References

Ablain, J., Durand, E.M., Yang, S., Zhou, Y., Zon, L.I., 2015. A CRISPR/Cas9 vector system for tissue-specific gene disruption in zebrafish. Developmental cell 32, 756-764.
Campbell, P.D., Heim, A.E., Smith, M.Z., Marlow, F.L., 2015. Kinesin-1 interacts with Bucky ball to form germ cells and is required to pattern the zebrafish body axis. Development 142, 2996-3008.
Gagnon, J.A., Valen, E., Thyme, S.B., Huang, P., Ahkmetova, L., Pauli, A., Montague, T.G., Zimmerman, S., Richter, C., Schier, A.F., 2014a. Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. PloS one 9, e98186.
Gagnon, J.A., Valen, E., Thyme, S.B., Huang, P., Akhmetova, L., Pauli, A., Montague, T.G., Zimmerman, S., Richter, C., Schier, A.F., 2014b. Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. PloS one 9, e98186.
Hartley, J.L., Temple, G.F., Brasch, M.A., 2000. DNA cloning using in vitro site-specific recombination. Genome research 10, 1788-1795.
Heim, A.E., Hartung, O., Rothhamel, S., Ferreira, E., Jenny, A., Marlow, F.L., 2014. Oocyte polarity requires a Bucky ball-dependent feedback amplification loop. Development (Cambridge, England) 141, 842-854.
Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V., Li, Y., Fine, E.J., Wu, X., Shalem, O., Cradick, T.J., Marraffini, L.A., Bao, G., Zhang, F., 2013. DNA targeting specificity of RNA-guided Cas9 nucleases. Nat Biotechnol 31, 827-832.
Hwang, W.Y., Fu, Y., Reyon, D., Maeder, M.L., Tsai, S.Q., Sander, J.D., Peterson, R.T., Yeh, J.R., Joung, J.K., 2013. Efficient genome editing in zebrafish using a CRISPR-Cas system. Nature biotechnology 31, 227-229.
Kawakami, K., Takeda, H., Kawakami, N., Kobayashi, M., Matsuda, N., Mishina, M., 2004. A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish. Developmental cell 7, 133-144.
Kim, H.J., Lee, H.J., Kim, H., Cho, S.W., Kim, J.S., 2009. Targeted genome editing in human cells with zinc finger nucleases constructed via modular assembly. Genome Res 19, 1279-1288.
Kwan, K.M., Fujimoto, E., Grabher, C., Mangum, B.D., Hardy, M.E., Campbell, D.S., Parant, J.M., Yost, H.J., Kanki, J.P., Chien, C.B., 2007. The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. Developmental dynamics : an official publication of the American Association of Anatomists 236, 3088-3099.
Leu, D.H., Draper, B.W., 2010. The ziwi promoter drives germline-specific gene expression in zebrafish. Developmental dynamics : an official publication of the American Association of Anatomists 239, 2714-2721.
Meeker, N.D., Hutchinson, S.A., Ho, L., Trede, N.S., 2007. Method for isolation of PCR-ready genomic DNA from zebrafish tissues. BioTechniques 43, 610, 612, 614.
Montague, T.G., Cruz, J.M., Gagnon, J.A., Church, G.M., Valen, E., 2014. CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. Nucleic acids research 42, W401-407.