© 2016. Published by The Company of Biologists Ltd | Development (2016) 143, 2868-2875 doi:10.1242/dev.138057

TECHNIQUES AND RESOURCES

RESEARCH ARTICLE

Leapfrogging: primordial germ cell transplantation permits recovery of CRISPR/Cas9-induced mutations in essential genes

Ira L. Blitz*, Margaret B. Fish and Ken W. Y. Cho

ABSTRACT

CRISPR/Cas9 genome editing is revolutionizing genetic loss-of-function analysis but technical limitations remain that slow progress when creating mutant lines. First, in conventional genetic breeding schemes, mosaic founder animals carrying mutant alleles are outcrossed to produce F1 heterozygotes. Phenotypic analysis occurs in the F2 generation following F1 intercrosses. Thus, mutant analyses will require multi-generational studies. Second, when targeting essential genes, efficient mutagenesis of founders is often lethal, preventing the acquisition of mature animals. Reducing mutagenesis levels may improve founder survival, but results in lower, more variable rates of germline transmission. Therefore, an efficient approach to study lethal mutations would be useful. To overcome these shortfalls, we introduce 'leapfrogging', a method combining efficient CRISPR mutagenesis with transplantation of mutated primordial germ cells into a wild-type host. Tested using *Xenopus tropicalis*, we show that founders containing transplants transmit mutant alleles with high efficiency. F1 offspring from intercrosses between F0 animals that carry embryonic lethal alleles recapitulate loss-of-function phenotypes, circumventing an entire generation of breeding. We anticipate that leapfrogging will be transferable to other species.

KEY WORDS: CRISPR/Cas9, TALENs, Knockouts, Primordial germ cells, Genome editing, *Xenopus*

INTRODUCTION

The use of CRISPR/Cas9 and TALEN programmable nucleases is revolutionizing genetic analyses and has been applied to a remarkable number of different organisms. However, the production of founder organisms carrying gene disruptions to produce mutants for loss of function (LOF) analysis has its challenges. The efficient mutagenesis of essential genes can result in lethality in the F0 generation and therefore failure to transmit mutant alleles to subsequent generations. Genetic screens in mouse and zebrafish have estimated that as many as 30% of genes are embryonic lethal (Driever et al., 1996; Haffter et al., 1996; Ayadi et al., 2012). Therefore, improvements in current genetic tools and/or manipulations to circumvent the lethality associated with mutation of essential genes would greatly accelerate progress in making mutant lines.

We and others have shown that programmable nucleases are efficient genome editing tools in the human disease model *Xenopus*, both in the diploid frog *Xenopus tropicalis* and its close allotetraploid relative *Xenopus laevis* (Young et al., 2011; Ishibashi et al., 2012; Lei et al., 2012; Blitz et al., 2013; Nakayama et al., 2013; Guo et al., 2014; Nakajima and Yaoita, 2015a; Wang et al., 2015). In an attempt to circumvent founder lethality, we sought to develop a method to confine targeted gene mutations to the germline, thereby 'protecting' somatic tissues from the deleterious effects of LOF mutations. Under such conditions, we expect that germ cells harboring specific mutations will successfully mature in healthy host animals that could transmit mutant alleles at high frequency to the F1 generation. Here, we present leapfrogging, which combines whole-embryo mutagenesis with transplantation of mutant primordial germ cells (PGCs) into wild-type sibling embryos.

Our approach was stimulated by studies in the early 1960s by Blackler and colleagues, who showed that transplantation of *Xenopus* posterior ventral flank from neurula or early tailbud stage embryos can confer the donor germline to recipient embryos, bolstering the idea that germ plasm-bearing cells establish the germline (Blackler, 1960; Blackler and Fischberg, 1961). We aimed to develop a more efficient transplantation procedure, combined with CRISPR/Cas9 mutagenesis, to accelerate research on identifying the functions of thousands of embryonic lethal genes. In *Xenopus* and other anurans, germplasm is first localized in the vegetal pole of the egg and early embryo, which is a more accessible position for both ablation and transplantation. Germline ablation has been partially or completely achieved by either vegetal UV irradiation or by cytoplasmic extrusion following pricking of the zygote's vegetal pole (Buehr and Blackler, 1970; Nieuwkoop and Sutasurya, 1979). During the early cleavages following fertilization, the germ plasm gradually coalesces into a small number of cells located near the vegetal pole (reviewed in Nieuwkoop and Sutasurya, 1979; Houston and King, 2000a). Leapfrogging combines efficient F0 embryo-wide mutagenesis with transplantation of mutation-bearing PGCs to wild-type hosts that have had their endogenous PGCs removed. We show that transplantation is readily achieved at late blastula stages when the PGCs are still in the vegetal-most domain. Leapfrogging results in efficient transmission of mutant alleles to F1 offspring, demonstrating successful transfer. We also demonstrate that embryonic lethal *goosecoid* (*gsc*) mutants can be analyzed for phenotypes in the F1 generation by intercrossing leapfrogged F0 adults. We anticipate that leapfrogging will accelerate CRISPR/Cas9- and TALEN-based genetic analyses in *Xenopus* and similar approaches may be adapted to a variety of organisms where programmable nucleases can be applied.

RESULTS

Blastula stage engraftment of presumptive PGCs

We first examined the efficacy of extirpating germ plasm-bearing cells by simple removal of vegetal explants. Late blastula (stage 9)

4410 Natural Sciences Building 2, Department of Developmental and Cell Biology, University of California, Irvine, CA 92697, USA.

*Author for correspondence (ilblitz@uci.edu)

I.L.B., 0000-0002-0173-3287

Received 29 March 2016; Accepted 15 June 2016

Development (2016) 143, 2868-2875 doi:10.1242/dev.138057

embryos were dissected between ~5 and 6 h post-fertilization (hpf), before the vegetal movements of gastrulation begin at 6.5 hpf (Fig. 1A). Vegetal explants and the embryo remainders ('carcasses') were subjected to whole-mount *in situ* hybridization to visualize expression of *deleted in azoospermia like* (*dazl*) mRNA (Houston et al., 1998; Houston and King, 2000b; Sekizaki et al., 2004), a marker for PGC localization (Fig. 1B). All vegetal explants contained numerous *dazl* expression foci (Fig. 1C), suggesting that their transplantation would efficiently shuttle donor PGCs to recipient embryos. Most carcasses (90%; 18/20) showed nearly complete removal of *dazl* signal (compare Fig. 1B and D, left). A small fraction of carcasses (2/20) showed a faint *dazl* signal (Fig. 1D, right). We conclude that our procedure can effectively remove the majority of PGCs from embryos.

Efficient CRISPR/Cas9 mutagenesis in PGC transplants

Since we wished to combine CRISPR/Cas9 mutagenesis with PGC transplantation, we sought an indirect assay for determining the efficacy of mutagenesis in the PGC-containing transplanted tissues, by using the remaining embryo carcass as a proxy for these cells. However, since the diffusibility of Cas9 ribonucleoprotein complexes in the early cytoplasm might be limited, it remained possible that the carcass might not reflect mutagenesis of PGCs in the vegetal pole. Therefore, we first assessed whether mutagenesis in the carcass is a reasonable approximation of the efficacy of mutagenesis within the PGC transplant. We injected Cas9-sgRNA complexes that target the gene *tyrosinase* (*tyr*), which results in the non-lethal albino phenotype when biallelically mutated, into the animal pole at the one-cell stage. Late blastula stage 9 embryos were then dissected, subdividing the embryo into PGC explant, animal cap and the remaining endomesodermal tissues (Fig. S1). The extent of mutagenesis was assessed in these explants by direct sequencing of PCR amplicons (DSP; Nakayama et al., 2014) containing the targeted region in *tyr*. Sequencing traces for populations of amplicons show a mixing of peaks beginning in the vicinity of the cleavage site, providing a rough measure of mutational efficacy. We found that all three dissected domains show similar DSP traces, suggesting that animal pole injections result in efficient mutagenesis in the vegetal-most PGC-containing explants. Therefore, we routinely use DSP on carcasses to verify the efficacy of mutagenesis in transplant-bearing animals before raising them to adulthood.

Efficient germline transmission by wild-type frogs carrying *tyr* mutant gametes

Of an original 30 *tyr* 'leapfrogged' embryos, 17 successfully passed through metamorphosis to froglet stages, and the first 10 to reach sexual maturity were assayed for germline transmission of *tyr* mutant alleles. F0 animals were crossed with animals from a homozygous albino ($tyr^{-/-}$) population that we previously established. Since the albino phenotype is only observed in homozygous *tyr*-deficient (null) offspring, scoring of the F1 animals for this phenotype effectively assayed the rate of mutant alleles transmitted by the gametes of leapfrogged animals. Both male and female animals were test crossed in this manner (Fig. 2A,B) and the results from these 10 crosses (with over 3500 offspring scored) are displayed in Table 1. Six of the animals bearing leapfrog transplants, representing both sexes, showed a remarkable rate of 100% germline transmission of mutant alleles (e.g. Fig. 2C). Three of the four remaining test crosses resulted in no albino embryos whereas the fourth had 41% transmission of mutant alleles. There are several possible explanations for the cases where low *tyr* transmission rates were observed. First, mutagenesis in some F0 donor embryos might have been very low. Since we confirm the efficacy of mutagenesis using a sequencing-based assay, this possibility is unlikely. Second, in some cases, the removal of the endogenous wild-type PGCs may have been insufficient, prior to transplantation of mutated PGCs. Lastly, the PGC transplant tissue may have been largely or completely 'expelled' from the embryo as a result of insufficient healing. Despite these possibilities, the high frequency of $tyr^{-/-}$ embryos generated demonstrates that leapfrogging results in efficient transfer of mutant PGCs into somatically wild-type animals.

Use of leapfrogging to recover embryonic lethal *goosecoid* mutant phenotypes in F1 embryos

Using albinism to assay for gametes carrying *tyr* alleles provided a rapid and easy high-throughput assay for germline transmission of mutant alleles, but these experiments do not demonstrate that leapfrogging permits the recovery of mutations in an essential gene.

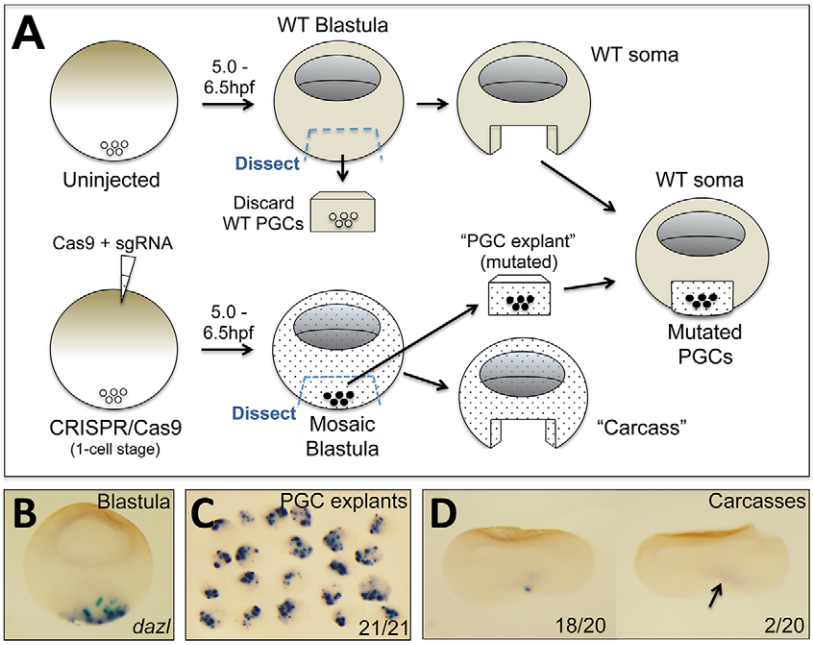

Fig. 1. Transplantation of PGCs. (A) Scheme for transplanting PGCs from CRISPR/Cas9-mutagenized blastula stage embryos (bottom) into a wild-type soma (top) that has had its PGCs removed. (B) Wild-type blastula showing vegetal localization of PGCs as detected by *dazl in situ* hybridization. (C) PGC explants show many foci of *dazl* expression. (D) Carcasses from blastula embryos show vastly reduced *dazl* expression foci, suggesting effective removal of PGCs.

Development (2016) 143, 2868-2875 doi:10.1242/dev.138057

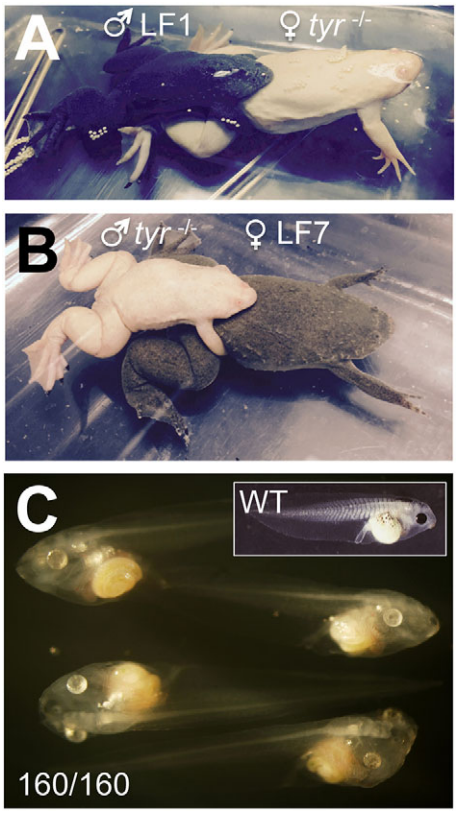

Fig. 2. Test crosses between animals carrying *tyr*-mutated leapfrog transplants and albinos demonstrate germline transmission of mutant alleles. (A) Leapfrog transplant-bearing male (pigmented) is shown amplexed with an albino $tyr^{-/-}$ female. (B) Leapfrog transplant-bearing female (pigmented) is shown amplexed with an albino $tyr^{-/-}$ male. (C) Examples of F1 progeny from the cross in A grown to tadpole stage. These tadpoles are albino because they inherited *tyr* mutant alleles from both F0 parents. Therefore the leapfrog-generated frog carries gametes derived from CRISPR-mutated PGCs. The inset in C shows an unrelated pigmented tadpole at roughly the same stage for comparison.

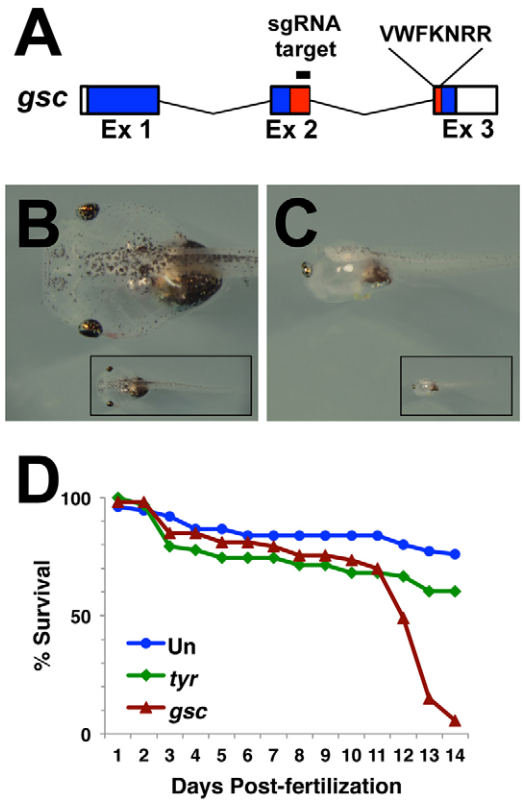

Fig. 3. Whole-animal targeting of *gsc* causes a dramatic reduction in survival in F0 embryos. (A) The *gsc* gene structure is shown. The open reading frame (ORF) is shown in blue and the homeobox, in red, is split between exons 2 and 3, with the DNA recognition helix (VWFKNRR) coding sequence found downstream of the exon 3 splice acceptor. The CRISPR/Cas9 target site location is indicated. (B) Representative wild-type (uninjected) tadpole and (C) a *gsc* CRISPR-injected cyclopic tadpole at 9 dpf illustrate the extent of defects in head/craniofacial development. Insets show whole tadpoles. Tadpoles are shown at the same magnification, as are insets. (D) A survival curve shows that the population of *gsc* targeted F0 embryos is severely reduced by 14 dpf. Plots for uninjected siblings (Un) and *tyr* CRISPR-injected embryos are shown as controls. Equivalent amounts of *gsc* and *tyr* sgRNAs were used.

Therefore we applied leapfrogging to a gene that displays a well-known embryonic lethal mutant phenotype, *gsc*, as a test case. *gsc* encodes the Goosecoid homeodomain transcription factor, which was identified in *Xenopus* based on its early gastrula stage expression in Spemann's organizer (Blumberg et al., 1991; Cho et al., 1991). Morpholino antisense oligonucleotide-mediated inhibition of *gsc* mRNA translation in *Xenopus* severely reduces development of the anterior head (Sander et al., 2007). We synthesized an sgRNA targeting a sequence within the homeobox, near the splice donor site in exon 2 (Fig. 3A). Since this site is just upstream of the coding sequence for the VWFKNRR motif of the DNA recognition helix encoded by exon 3, we expected that any mutation at the target site, including single in-frame codon deletions or insertions, would disrupt proper folding of the DNA-binding domain, thereby resulting in null alleles.

Table 1. Phenotypic scoring of F1 progeny derived from test crosses of animals bearing leapfrog transplant carrying *tyr*-mutated PGCs

Cross	Male	Female	Albino	WT	Total	% Albino
1	LF1	$tyr^{-/-}$	160	0	160	100
2	LF2	$tyr^{-/-}$	148	0	148	100
3	$tyr^{-/-}$	LF3	409	0	409	100
4	$tyr^{-/-}$	LF4	0	519	519	0
5	$tyr^{-/-}$	LF5	223	0	223	100
6	LF6	$tyr^{-/-}$	493	703	1196	41.2
7	$tyr^{-/-}$	LF7	1	94	95	1.1
8	$tyr^{-/-}$	LF8	125	0	125	100
9	$tyr^{-/-}$	LF9	0	417	417	0
10	$tyr^{-/-}$	LF10	252	0	252	100

Entries in 'Male' and 'Female' columns indicate which of the parents in each cross contributes only tyr^{-} alleles and which is derived from leapfrogging (LF).

Preliminary testing of the *gsc* sgRNA in F0 embryos showed varying degrees of loss of anterior head tissue, including cyclopia (Fig. 3B,C; data not shown) and was accompanied by high lethality. A large and reproducible population decline occurs in the second week of development (Fig. 3D). We hypothesize that these tadpoles die from starvation as a result of defects in mouth and/or pharyngeal structures or from cardiac defects (Yamada et al., 1995; Rivera-Pérez et al., 1995; Filosa et al., 1997). Interestingly, even apparently phenotypically wild-type F0 animals (presumably less mutagenized) that survive this initial lethality show reduced overall body size as froglets compared with their uninjected siblings and continue to expire as immature adults (data not shown), illustrating the challenges in raising such F0 founder animals using conventional breeding schemes.

Since mutagenesis in F0 embryos appeared to be efficient from DSP assays, we created F0 animals bearing PGC transplants from

Development (2016) 143, 2868-2875 doi:10.1242/dev.138057

gsc CRISPR/Cas9-injected siblings. When individuals from both sexes reached sexual maturity, an intercross of leapfrogged animals was performed. Eighty-five embryos were produced from the first mating and embryos were allowed to develop to early tailbud stage for morphological and molecular analyses. We found that 73% of embryos developed with moderate to severe anterior head truncations while 27% appeared phenotypically wild type. Nine morphologically wild-type embryos and 15 presumptive mutants were fixed at early tailbud stage 29/30 and these were subjected to whole-mount *in situ* hybridization with a probe cocktail to detect *otx2*, *egr2* (formerly *krox20*) and *hoxb9* (Papalopulu et al., 1991; Lamb et al., 1993; Godsave et al., 1994; Blitz and Cho, 1995; Pannese et al., 1995). Mutant embryos show loss of the anterior portion of the *otx2* expression domain without effects on more posterior neural expression (Fig. 4A-C; Fig. S2) in a pattern virtually identical to that previously observed in *gsc* morpholino knockdown experiments in *X. laevis* (Sander et al., 2007).

A second mating using the same pair produced 370 embryos, which were grown to tailbud stage 40 to assess the extent of anterior truncation morphologically (Fig. 4D-F). This second mating produced a similar ratio of 68% mutant to 32% wild type. Interestingly, of the 252 embryos showing head truncations, 135 were cyclopic (Fig. 4E) whereas 117 were headless (Fig. 4F). Since our mutagenesis strategy targeted the DNA-binding domain to ensure LOF, the most parsimonious explanation is that the LOF effect on anterior development is variably penetrant.

To correlate the phenotype with mutations at the *gsc* target site, we subsequently genotyped both phenotypically wild-type and mutant embryos. Nearly all (15/16) phenotypically wild-type embryos were heterozygotes, with one being homozygous wild type (Fig. S3). In contrast, 100% of phenotypically mutant embryos carried biallelic *gsc* mutations with indels around the target site (Fig. S3). Because all of the observed mutations disrupt the coding sequence of the Gsc homeodomain (Figs S3 and S4), and all but one remove the DNA recognition helix entirely, we anticipate these would abrogate DNA binding, thereby resulting in null alleles. We conclude that there is a 100% correspondence between biallelic *gsc* LOF genotypes and mutant phenotypes.

In summary, leapfrogging mitigates founder lethality when targeting essential genes and a high percentage of homozygous mutant F1 animals can be generated for subsequent characterization of non-mosaic LOF phenotypes.

DISCUSSION

Methods for creating mutant lines are needed that can mitigate lethality in F0 animals when mutating essential genes while retaining efficient germline transmission. Several potential solutions to this problem have been published. In rodents, the introduction of programmable nucleases, performed either *in vivo* or *in vitro*, into adult spermatogonial or oogonial stem cells has been accomplished (Fanslow et al., 2014; Chapman et al., 2015; Sato et al., 2015; Takahashi et al., 2015; Wu et al., 2015). These mutated germ cell precursors variably contribute to the germline while endogenous unmutated germ cells remain. Significant technology development, especially to target both sexes, would be required to successfully apply these methods to new systems. A second approach is to enrich Cas9 or TALEN mRNAs in the germ plasm using 3′ UTRs derived from germ plasm-localized mRNAs such as *nanos1* or *ddx25* (Moreno-Mateos et al., 2015; Nakajima and Yaoita, 2015b). Since successful partitioning to the germ plasm requires careful titration to minimize targeting of somatic nuclei, the frequency of germline transmission is highly variable. The use of 3′ UTRs to drive Cas9 mRNA into germ cells has not been demonstrated in *Xenopus*, but has been used in zebrafish (Moreno-Mateos et al., 2015). However, we speculate that the use of highly efficient doses of Cas9-sgRNA complexes or efficient doses of Cas9 mRNA plus sgRNA (see Nakayama et al., 2014) might have an additional advantage over titrated low doses of Cas9-3′UTR fusion mRNAs. The former approach is expected to yield mutations at earlier stages of development (Bhattacharya et al., 2015), resulting in a smaller diversity of alleles being transmitted in the F0 leapfrogged germline. In our *gsc* leapfrogging experiment, we recovered 10 different mutant alleles from the 37 F1 embryos analyzed (Fig. S3). The most parsimonious explanation for the allelic combinations found is that the number of mutant alleles carried by each parent is probably between 4 and 8 (see Fig. S3C). It would be valuable to directly compare the efficacy of each approach in the future by creating F0 lines.

While we used CRISPR/Cas9 to mutagenize PGCs, we believe that TALENs can also be successfully applied to generate efficient

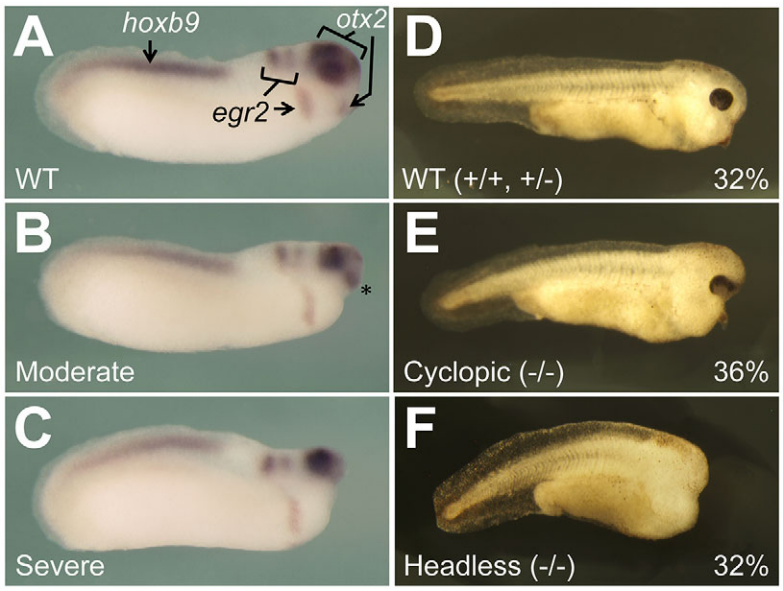

Fig. 4. F1 embryos derived from intercrosses of F0 *gsc* leapfrogged adults show variable loss of anterior head structures. (A-C) Whole-mount *in situ* hybridization of F0 embryos using a cocktail of riboprobes for *otx2*, *egr2* and *hoxb9* marking increasingly posterior domains of the embryo. Loss of the anterior portion of the *otx2* expression domain is seen (note region marked with asterisk in B that is not readily distinguishable in the embryo in C), while the more posterior expression domains remain unaffected. (D-F) From a second mating, 370 embryos were grown to mid-tailbud stage 40 to assess the severity of loss of anterior head structures. In approximately one-third of the embryos (E), eyes fuse in the anterior midline whereas in another third (F) a more severe anterior truncation is seen and eye structures fail to form.

Development (2016) 143, 2868-2875 doi:10.1242/dev.138057

mutagenesis using leapfrogging. The use of PGCs allows for a single technology to produce both male and female F0 animals with a high percentage of their donor-derived gametes bearing mutant alleles. Since the recipient of these modified PGCs is somatically wild type, transplant-bearing animals are viable and only carry mutations in germ cells. Intercrossing of leapfrogged animals is capable of producing a high frequency of mutants. Our experiments with *tyr* suggest that germline transmission rates can frequently be 100%. This high efficiency of germline transmission is especially advantageous if one needs large amounts of biological material (i.e. egg or embryo extracts and ChIP-seq, where genotyping of hundreds to thousands of individual embryos would be prohibitive), from mutant embryos. This is a major strength of the *Xenopus* system. Since leapfrogging produces non-mosaic phenotypic animals in the F1 generation, this method circumvents a full generation of time-consuming and laborious screening (e.g. test crosses of numerous F1 animals to find those showing germline transmission) required with the F2 analysis of the more standard breeding regimen.

It is useful to note that leapfrogging also offers a new method to study the function of maternally expressed genes. Recent studies have shown that maternal RNAs are present until late gastrulation (Owens et al., 2016) and maternally deposited proteins may also persist until at least tailbud stage 33 (Peshkin et al., 2015). This indicates that maternal gene products might play a much larger role in embryonic development than previously recognized, and that a great deal of biology in the early embryo may be refractory to F0 analysis using genome editing, therefore requiring genetic crosses. A successful approach to LOF analysis of maternal gene products utilizes the host transfer method (Heasman et al., 1991; Olson et al., 2012). Stabilized antisense oligonucleotides act in conjunction with endogenous RNaseH in oocytes to target and degrade maternal RNAs during *in vitro* oocyte maturation. Manipulated oocytes are surgically reintroduced into adult females that provide the jelly coat needed for fertilization. While this approach has been successful in revealing the functions of maternal gene products, it can be technically challenging and often produces incomplete knockdowns. In summary, leapfrogging provides a genetic method for F1 analysis of maternal gene knockouts. Additionally, only a leapfrogged female is required for these analyses as the maternal products are provided in the eggs. Fertilization with wild-type sperm would permit assessment of loss of maternal gene function. We note that in cases where the targeted gene is required for the production of germ cells, leapfrogging might be difficult or impossible to achieve, whereas the host transfer method may be more advantageous as it allows for knockdowns after oogenesis is complete.

Factors influencing successful leapfrogging

To achieve a high percentage of mutant (LOF) alleles in the germline of F0 animals by leapfrogging, there are a number of important considerations. First, it is essential that a high percentage of genomes in the donor PGCs bear mutations in the targeted gene. A number of recommendations for efficient CRISPR/Cas9-mediated mutagenesis in *Xenopus tropicalis* are published (Nakayama et al., 2014). We find that the most critical determinants for efficient CRISPR/Cas9 mutagenesis in *Xenopus* are injection of optimized quantities of Cas9-sgRNA complexes, combined with the selection of efficient sgRNAs. Careful dose optimization and preliminary testing of a handful of sgRNA target sites is recommended prior to transplantation experiments.

Second, careful determination of the location of sgRNA target sites within a gene is essential. A common strategy is to target a site near the 5′ end of the ORF, with the expectation that indels will result in frameshift mutations to block protein translation. While this might be a good strategy for making mutants via the standard three-generation breeding scheme, this approach is poorly suited for leapfrogging. Two-thirds of the alleles from repair of double-strand breaks within an ORF are expected to result in frameshifts, but one-third of the alleles will be in-frame indels (usually small deletions). This expectation has been experimentally verified on a large scale in zebrafish embryos, where 68% of indels were found to result in frameshifts and 32% were in-frame deletions, with >75% being smaller than 12 bp deletions (Varshney et al., 2015). Therefore, a significant number of mutant alleles are expected to have either wild-type levels of activity or be hypomorphic, and thus not complete LOF. F1 animals resulting from such F0 intercrosses would be ineffective for displaying mutant phenotypes. For leapfrogging to be maximally successful, all mutant alleles need to be strong LOF mutations. We recommend selecting sgRNA target sites within the coding regions of recognizable protein folding domains so that even in-frame indels will display defects. In the case of *gsc*, by targeting within the DNA-binding domain, in-frame deletions will result in misfolding and loss of DNA binding capability. Therefore, careful target choice results in ∼100% of indels being LOF mutants. This principle of targeting folded protein domains can be applied to other classes of protein-coding genes, an approach validated by a recent study (Shi et al., 2015). Numerous sites were targeted across a number of chromatin regulatory genes to scan for optimal loss of protein function and a higher proportion of null mutations were found when targeting folded domains.

Third, for successful leapfrogging, efficient removal of PGCs from the recipient embryo must also accompany transplantation of well-mutagenized donor PGCs. When we examined our PGC extirpation performed at blastula stages, a minority of *dazl*-stained foci were found (Fig. 1). These remaining wild-type PGCs might contribute to the variable rates of transmission of mutant alleles that we see. An alternative interpretation is that the deep cells positive for residual *dazl* expression in the vegetal yolk mass (Fig. 1B) may not represent future PGCs. It has been suggested that microRNAs contribute to the clearing of germ plasm transcripts from somatic cells that do not contribute to the PGCs (Koebernick et al., 2010; Yang et al., 2015). In the future it would be interesting to deplete PGCs in host embryos to determine whether this results in increased frequency of mutant alleles in the 'leapfrogged' germline. This can be achieved by morpholino knockdowns of *ddx25* (also known as *deadsouth*), *nanos1* or *dnd1* (also known as *dead end*), all of which have been shown to deplete PGCs (Horvay et al., 2006; Lai et al., 2012; Yamaguchi et al., 2013). This approach may permit the use of smaller tissue fragments for transplantation, thus further minimizing the likelihood of causing physical damage caused by transplantation, and also reducing endodermal carryover.

Lastly, we recommend producing a number of transplant-bearing animals, both males and females, so that these sufficient numbers are available to survive to sexual maturity. In our various transplantation experiments, 50-75% of leapfrogged embryos survive to adulthood. Further investigation into the size of transplants required for successful leapfrogging should be tested as smaller transplants might improve survival rates. Modification of other conditions for transplantation might increase our success further. For example, recent testing of conditions for successful PGC transplantation in *Xenopus laevis* revealed that a higher 'strength' embryo culture solution [1× Marc's modified Ringer solution (MMR)] is required for efficient healing (our unpublished

Development (2016) 143, 2868-2875 doi:10.1242/dev.138057

observations) than we used in the current study for *Xenopus tropicalis*.

Can leapfrogging advance genetic manipulations in other organisms?

Combining programmable nuclease-mediated genome editing with transplantation of PGCs should be applicable to many other animals. PGC transplantation has been performed in a number of species and therefore the only barrier to using leapfrogging is an efficient method for delivery and mutagenesis by programmable nucleases. It is likely that transplantation of PGCs can be performed successfully in other animals that (like *Xenopus*) have a maternally localized germ plasm, such as the sturgeon (Saito et al., 2014), which is a fish of economic importance. Teleost fish such as zebrafish, medaka, goby and others also have maternally deposited germ plasm, although it is initially not confined to a single location (Yoon et al., 1997; Miyake et al., 2006; Herpin et al., 2007). However, PGCs have been both directly transplanted between zebrafish embryos and also after growth in cell culture (Ciruna et al., 2002; Kawakami et al., 2010), making leapfrogging feasible in this model system. In other animals, the PGCs are not maternally derived but instead are specified by an inductive mechanism. PGC transplantation has also been demonstrated in several of these species. For example, transplantation of ventral marginal zone between early gastrula stage embryos in caudate amphibians (salamanders and newts) such as the Mexican axolotl *Amblystoma mexicanum* results in transfer of the germline between animals (Nieuwkoop, 1947; Smith, 1964; Chatfield et al., 2014). Leapfrogging might also be viable in birds. PGCs have been transplanted from cultured early chick extraembryonic tissue and can contribute to the germline in recipients (van de Lavoir et al., 2006; Nakamura et al., 2013). We envision combining this technology with programmable nucleases (Park et al., 2014; Véron et al., 2015) to make leapfrogging possible in this and a number of other species.

MATERIALS AND METHODS

Vegetal explants and transplantation

Vegetal explants were prepared from *X. tropicalis* embryos between early stage 9 and the first appearance of bottle cells at stage 10. These developmental stages, using Nieuwkoop and Faber staging for *Xenopus laevis* (Nieuwkoop and Faber, 1967), are roughly 5-6.5 hpf when embryos are cultured at 24-25°C (Owens et al., 2016). Explants were prepared using eyebrow hair knives and hair loops and dissections were performed in agarose-coated 60 mm plastic dishes in 0.3× MMR (Sive et al., 2000) containing gentamycin. Embryos were first dechorionated with Dumond watchmaker's forceps and explants measured ∼0.4-0.45 mm in width and ∼0.25-0.3 mm in depth, which corresponds to approximately 1/2 to 2/3 the distance from the vegetal pole to the blastocoel floor. These dimensions were used to maximize extirpation of germ plasm-bearing cells from recipient embryos. Vegetal tissue was first dissected from recipient embryos, which were set aside while graft donor embryos were dissected. Once donor tissues were isolated, they were quickly placed into the recipient's open vegetal wound with the interior surface of the graft being placed into the wound. Gentle pressure was applied to place the graft securely and the recipient embryo was moved to a well cut into the agarose to allow for healing while the next transplantation was being performed. Donor embryo 'carcasses' were similarly moved to wells. Typically ∼12 transplants can be accomplished during the 90 min time window afforded. Once grafts had healed into place, embryos were carefully placed, vegetal pole up, into individual agarose-coated wells in 12-well plates containing 1/9× MMR plus gentamycin. Donor carcasses were also moved to individual wells in the 12-well plates and care was taken to keep track of carcasses and the corresponding embryos receiving grafts. All embryos were subsequently cultured overnight in a 25°C incubator. Carcasses were typically homogenized in proteinase K-containing lysis buffer the following day to provide material for DSP analysis (Nakayama et al., 2014) to verify the success of CRISPR/Cas9 mutagenesis in DNA of the corresponding grafts.

CRISPR/Cas9 mutagenesis

Synchronous embryos were obtained by *in vitro* fertilization, dejellied at 10 min post-fertilization by mild agitation in 3% cysteine (pH 7.6-7.8) and transferred to agarose-coated plates containing 1/9× MMR. Prior to injections, a cocktail of Cas9 protein (PNA Bio) and *tyr* sgRNA (Blitz et al., 2013) was prepared by preincubating the sgRNA at 60°C for 5 min. This was quick-cooled on ice and 1 µl Cas9 (1 µg/µl in 20 mM HEPES pH 7.5, 150 mM KCl and 1% sucrose) was added. The cocktail was mixed by gentle tapping and then incubated at 37°C for 10 min. One-cell stage embryos were microinjected into a single site at the animal pole with 4 nl of Cas9/sgRNA cocktail, receiving a total of 1 ng Cas9 and 250 pg *tyr* sgRNA. Embryos were moved to agarose-coated plates and cultured in 1/9× MMR at 25°C until early stage 9 (∼4.5 hpf). Embryos were dissected between ∼5 hpf and ∼6.5 hpf (stage 10).

To create a template for transcribing *gsc* targeting sgRNA [target site: CCTCAGAGAGGAAAAAGTAGagg, with the protospacer adjacent motif (PAM) in lower case], we designed two overlapping oligonucleotides as follows. A 'top strand' 62 nt oligo, 5′-TAATACGACTCACTATAGG-[CCTCAGAGAGGAAAAAGTAG]GTTTTAGAGCTAGAAATAGCAA-G-3′, was designed containing a T7 RNA promoter (underlined), followed by the target sequence (in brackets and without the PAM) and 23 nt of the guide backbone. A second universal bottom strand oligo of 80 nt in length, 5′-AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGA-CTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC-3′ with 23 nt overlap to the top oligo was used in the template assembly reaction as previously outlined (Nakayama et al., 2014). Following synthesis, the reaction was phenol/chloroform extracted, precipitated and resuspended in DEPC-treated H_2O. Guide RNA synthesis utilized 40-50 ng template in a 20 µl T7 Megascript (ThermoFisher) *in vitro* transcription reaction overnight at 37°C. The reaction was treated with Turbo DNaseI (ThermoFisher) and then phenol/chloroform extracted, precipitated with ammonium acetate and isopropanol, and resuspended in DEPC-treated H_2O. For efficient mutagenesis, we used 1 ng Cas9 protein (PNA Bio CP-01) and 1.2 ng of *gsc* sgRNA/embryo, which was precomplexed as described above. To perform survival comparisons between *gsc* and *tyr* sgRNA-injected embryos we used 1.2 ng *tyr* sgRNA/embryo.

Assessing CRISPR/Cas9 mutagenesis and genotyping of F1 animals

Embryo lysis and DSP assays were performed as described (Nakayama et al., 2014) using proofreading Pfx Platinum DNA polymerase (Invitrogen). Oligonucleotides for *tyr* amplications and sequencing were previously described (Blitz et al., 2013). For genotyping of *gsc* mutants, we used a combination of DSP assays and sequencing of individual cloned PCR products. Oligos for *gsc* target region amplification and sequencing were 5′-CCACACACATAAAGCTCCACAT-3′ and 5′-ACACATTTGGGCCCTG-GGTA-3′. Following PCR amplification, amplicons were cloned using a Zero Blunt Topo Cloning Kit (Invitrogen). Individual clones in pCRBluntII-TOPO were sequenced at Genewiz.

Whole-mount *in situ* hybridizations

To create digoxigenin-labeled probes, cDNA/EST and genomic sequence information for *X. tropicalis* was retrieved from Xenbase (www.xenbase.org; RRID:SCR_003280; Karpinka et al., 2015). Oligonucleotides were designed to PCR amplify an 877 bp fragment of *dazl* from genomic DNA with a bacteriophage T7 promoter added to the 5′ end of the 'reverse' strand as follows: forward strand, 5′-GGACGATAGTGTGCACCAATTCA-3′; reverse, 5′-GCAGCTAATACGACTCACTATAGGACCACAGATTGCCC-AGTGCT-3′. The T7 promoter is underlined and a 5 bp 5′ extension (Nakayama et al., 2014) was added to enhance *in vitro* transcription during riboprobe synthesis. This *dazl* DNA template was amplified from *X. tropicalis* liver genomic DNA using a touchdown strategy using Pfx polymerase (Invitrogen) with the following conditions: 94°C for 5 min, followed by 13

Development (2016) 143, 2868-2875 doi:10.1242/dev.138057

cycles of 94°C for 20 s, 65°C annealing for 20 s, 68°C extension for 1 min, with each cycle's annealing step decreasing the temperature by 0.5°C. This segment was followed by 30 cycles of 94°C for 20 s, 58°C annealing for 20 s, 68°C extension for 1 min, followed by a 5 min extension at 68°C. Similarly, templates were prepared by genomic PCR for *otx2*, *egr2* and *hoxb9*. PCR oligos were as follows. *otx2*: forward, 5′-CAGCAACAGCAGCAGC-AGAA-3′; reverse 5′-GCAGCTAATACGACTCACTATAGTTGCCAGAT-CCAGGGGAAAA-3′. *egr2*: forward, 5′-GCGATCGCTGGATTTCTCCT-3′; reverse, 5′-GCAGCTAATACGACTCACTATAGGCACTTGTGCCCAA-GCATTC-3′. *hoxb9*: forward, 5′-AACCCCTCAGCCAActggtta-3′; reverse, 5′-GCAGCTAATACGACTCACTATAGAAAGCGAGGGCGTT-TCTTGT-3′. Probe lengths were 1.6 kb (*otx2*), 1.5 kb (*egr2*) and 0.7 kb (*hoxb9*), respectively.

Whole-mount *in situ* hybridization was carried out according to Harland (1991) with modifications (Blitz and Cho, 1995). In addition, the protease permeabilization step used 2.5 µg proteinase K (Roche)/ml PTw (PBS containing 0.1% Tween 20) for 5 min. Hybridization steps were performed at 65°C and post-hybridization RNase digestion employed 1 µg RNaseA/ml and no RNase T1. Pigment was bleached post-staining according to Mayor et al. (1995). *dazl*-stained embryos were photographed after clearing in Murray's solution (2 benzyl benzoate: 1 benyzl alcohol). *otx2*, *egr2* and *hoxb9* triple-stained embryos were photographed in methanol.

Natural matings

Sexually mature animals were primed with 10 units of human chorionic gonadotropin (HCG; Chorulon) within a few days prior to boosting, which employed 100 units of HCG. Frogs were placed in 1/9× MMR and allowed to amplex. Frogs were removed the following day, gentamycin was added to the 1/9× MMR and embryos were kept at 25°C until hatching. Tadpoles were moved to clean 1/9× MMR with gentamycin until scoring for albinism after stage 41 or as described in the text for *gsc* phenotypes. Institutional IACUC guidelines were followed for all animal care and experimentation.

Note added in proof

Two important studies were recently published that demonstrate the viability of leapfrogging-like strategies in chicken (Oishi et al., 2016; Dimitrov et al., 2016).

Acknowledgements

The authors would like to thank Matt Guille and the European *Xenopus* Resource Center (RRID: SCR_007164) for an albino female, Takuya Nakayama, Arul Subramanian and Aaron Zorn for many valuable discussions. I.L.B. dedicates this paper to Dr Herman E. Brockman, an inspirational teacher, mentor, and tireless advocate for molecular genetics.

Competing interests

The authors declare no competing or financial interests.

Author contributions

I.L.B. and M.B.F. performed the experiments. All authors contributed to the design of the study and to writing of the manuscript.

Funding

This research was funded by the National Institute of Child Health and Human Development [5R21HD080684-02 to I.L.B.]. Deposited in PMC for release after 12 months.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.138057.supplemental

References

Ayadi, A., Birling, M.-C., Bottomley, J., Bussell, J., Fuchs, H., Fray, M., Gailus-Durner, V., Greenaway, S., Houghton, R., Karp, N. et al. (2012). Mouse large-scale phenotyping initiatives: overview of the European Mouse Disease Clinic (EUMODIC) and of the Wellcome Trust Sanger Institute Mouse Genetics Project. *Mamm. Genome* **23**, 600-610.

Bhattacharya, D., Marfo, C. A., Li, D., Lane, M. and Khokha, M. K. (2015). CRISPR/Cas9: an inexpensive, efficient loss of function tool to screen human disease genes in Xenopus. *Dev. Biol.* **408**, 196-204.

Blackler, A. W. (1960). Transfer of germ-cells in *Xenopus laevis*. *Nature* **185**, 859-860.

Blackler, A. W. and Fischberg, M. (1961). Transfer of primordial germ-cells in *Xenopus laevis*. *J. Embryol. Exp. Morph.* **9**, 634-641.

Blitz, I. L. and Cho, K. W. Y. (1995). Anterior neurectoderm is progressively induced during gastrulation: the role of the *Xenopus* homeobox gene *orthodenticle*. *Development* **121**, 993-1004.

Blitz, I. L., Biesinger, J., Xie, X. and Cho, K. W. Y. (2013). Biallelic genome modification in F_0*Xenopus tropicalis* embryos using the CRISPR/Cas system. *Genesis* **51**, 827-834.

Blumberg, B., Wright, C. V., De Robertis, E. M. and Cho, K. W. (1991). Organizer-specific homeobox genes in *Xenopus laevis* embryos. *Science* **253**, 194-196.

Buehr, M. L. and Blackler, A. W. (1970). Sterility and partial sterility in the South African clawed toad following the pricking of the egg. *J. Embryol. Exp. Morphol.* **23**, 375-384.

Chapman, K. M., Medrano, G. A., Jaichander, P., Chaudhary, J., Waits, A. E., Nobrega, M. A., Hotaling, J. M., Ober, C. and Hamra, F. K. (2015). Targeted germline modifications in rats using CRISPR/Cas9 and spermatogonial stem cells. *Cell Rep.* **10**, 1828-1835.

Chatfield, J., O'Reilly, M.-A., Bachvarova, R. F., Ferjentsik, Z., Redwood, C., Walmsley, M., Patient, R., Loose, M. and Johnson, A. D. (2014). Stochastic specification of primordial germ cells from mesoderm precursors in axolotl embryos. *Development* **141**, 2429-2440.

Cho, K. W. Y., Blumberg, B., Steinbeisser, H. and De Robertis, E. M. (1991). Molecular nature of Spemann's organizer: the role of the *Xenopus* homeobox gene *goosecoid*. *Cell* **67**, 1111-1120.

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.

Dimitrov, L., Pedersen, D., Ching, K. H., Yi, H., Collarini, E. J., Izquierdo, S., van de Lavoir, M. C. and Leighton, P. A. (2016). Germline gene editing in chickens by efficient CRISPR-mediated homologous recombination in primordial germ cells. *PLoS ONE* **11**, e0154303.

Driever, W., Solnica-Krezel, L., Schier, A. F., Neuhauss, S. C., Malicki, J., Stemple, D. L., Stainier, D. Y., Zwartkruis, F., Abdelilah, S., Rangini, Z. et al. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* **123**, 37-46.

Fanslow, D. A., Wirt, S. E., Barker, J. C., Connelly, J. P., Porteus, M. H. and Dann, C. T. (2014). Genome editing in mouse spermatogonial stem/progenitor cells using engineered nucleases. *PLoS ONE* **9**, e112652.

Filosa, S., Rivera-Pérez, J. A., Gómez, A. P., Gansmuller, A., Sasaki, H., Behringer, R. R. and Ang, S. L. (1997). Goosecoid and HNF-3beta genetically interact to regulate neural tube patterning during mouse embryogenesis. *Development* **124**, 2843-2854.

Godsave, S., Dekker, E.-J., Holling, T., Pannese, M., Boncinelli, E. and Durston, A. (1994). Expression patterns of Hoxb genes in the *Xenopus* embryo suggest roles in anteroposterior specification of the hindbrain and in dorsoventral patterning of the mesoderm. *Dev. Biol.* **166**, 465-476.

Guo, X., Zhang, T., Hu, Z., Zhang, Y., Shi, Z., Wang, Q., Cui, Y., Wang, F., Zhao, H. and Chen, Y. (2014). Efficient RNA/Cas9-mediated genome editing in *Xenopus tropicalis*. *Development* **141**, 707-714.

Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., van Eeden, F. J., Jiang, Y. J., Heisenberg, C. P. et al. (1996). The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development* **123**, 1-36.

Harland, R. M. (1991). In situ hybridization: an improved whole-mount method for Xenopus embryos. *Methods Cell Biol.* **36**, 685-695.

Heasman, J., Holwill, S. and Wylie, C. C. (1991). Fertilization of cultured *Xenopus* oocytes and use in studies of maternally inherited molecules. *Methods Cell Biol.* **36**, 213-230.

Herpin, A., Rohr, S., Riedel, D., Kluever, N., Raz, E. and Schartl, M. (2007). Specification of primordial germ cells in medaka (*Oryzias latipes*). *BMC Dev. Biol.* **7**, 3.

Horvay, K., Claussen, M., Katzer, M., Landgrebe, J. and Pieler, T. (2006). Xenopus Dead end mRNA is a localized maternal determinant that serves a conserved function in germ cell development. *Dev. Biol.* **291**, 1-11.

Houston, D. W. and King, M. L. (2000a). Germ plasm and molecular determinants of germ cell fate. *Curr. Top. Dev. Biol.* **50**, 155-181.

Houston, D. W. and King, M. L. (2000b). A critical role for *Xdazl*, a germ plasm-localized RNA, in the differentiation of primordial germ cells in *Xenopus*. *Development* **127**, 447-456.

Houston, D. W., Zhang, J., Maines, J. Z., Wasserman, S. A. and King, M. L. (1998). A *Xenopus* DAZ-like gene encodes an RNA component of germ plasm and is a functional homologue of *Drosophila boule*. *Development* **125**, 171-180.

Ishibashi, S., Cliffe, R. and Amaya, E. (2012). Highly efficient bi-allelic mutation rates using TALENs in *Xenopus tropicalis*. *Biol. Open* **1**, 1273-1276.

Karpinka, J. B., Fortriede, J. D., Burns, K. A., James-Zorn, C., Ponferrada, V. G., Lee, J., Karimi, K., Zorn, A. M. and Vize, P. D. (2015). Xenbase, the *Xenopus* model organism database; new virtualized system, data types and genomes. *Nucleic Acids Res.* **43**, D756-D763.

Kawakami, Y., Goto-Kazeto, R., Saito, T., Fujimoto, T., Higaki, S., Takahashi, Y., Arai, K. and Yamaha, E. (2010). Generation of germ-line chimera zebrafish using primordial germ cells isolated from cultured blastomeres and cryopreserved embryoids. *Int. J. Dev. Biol.* **54**, 1493-1501.
Koebernick, K., Loeber, J., Arthur, P. K., Tarbashevich, K. and Pieler, T. (2010). Elr-type proteins protect *Xenopus Dead end* mRNA from miR-18-mediated clearance in the soma. *Proc. Natl. Acad. Sci. USA* **107**, 16148-16153.
Lai, F., Singh, A. and King, M. L. (2012). Xenopus Nanos1 is required to prevent endoderm gene expression and apoptosis in primordial germ cells. *Development* **139**, 1476-1486.
Lamb, T. M., Knecht, A. K., Smith, W. C., Stachel, S. E., Economides, A. N., Stahl, N., Yancopolous, G. D. and Harland, R. M. (1993). Neural induction by the secreted polypeptide noggin. *Science* **262**, 713-718.
Lei, Y., Guo, X., Liu, Y., Cao, Y., Deng, Y., Chen, X., Cheng, C. H. K., Dawid, I. B., Chen, Y. and Zhao, H. (2012). Efficient targeted gene disruption in Xenopus embryos using engineered transcription activator-like effector nucleases (TALENs). *Proc. Natl. Acad. Sci. USA* **109**, 17484-17489.
Mayor, R., Morgan, R. and Sargent, M. G. (1995). Induction of the prospective neural crest of *Xenopus*. *Development* **121**, 767-777.
Miyake, A., Saito, T., Kashiwagi, T., Ando, D., Yamamoto, A., Suzuki, T., Nakatsuji, N. and Nakatsuji, T. (2006). Cloning and pattern of expression of the shiro-uo vasa gene during embryogenesis and its roles in PGC development. *Int. J. Dev. Biol.* **50**, 619-625.
Moreno-Mateos, M. A., Vejnar, C. E., Beaudoin, J.-D., Fernandez, J. P., Mis, E. K., Khokha, M. K. and Giraldez, A. J. (2015). CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting *in vivo*. *Nat. Methods* **12**, 982-988.
Nakajima, K. and Yaoita, Y. (2015a). Highly efficient gene knockout by injection of TALEN mRNAs into oocytes and host transfer in *Xenopus laevis*. *Biol. Open* **4**, 180-185.
Nakajima, K. and Yaoita, Y. (2015b). Development of a new approach for targeted gene editing in primordial germ cells using TALENs in *Xenopus*. *Biol. Open* **4**, 259-266.
Nakamura, Y., Kagami, H. and Tagami, T. (2013). Development, differentiation and manipulation of chicken germ cells. *Dev. Growth Differ.* **55**, 20-40.
Nakayama, T., Fish, M. B., Fisher, M., Oomen-Hajagos, J., Thomsen, G. H. and Grainger, R. M. (2013). Simple and efficient CRISPR/Cas9-mediated targeted mutagenesis in *Xenopus tropicalis*. *Genesis* **51**, 835-843.
Nakayama, T., Blitz, I. L., Fish, M. B., Odeleye, A. O., Manohar, S., Cho, K. W. Y. and Grainger, R. M. (2014). Cas9-based genome editing in *Xenopus tropicalis*. *Methods Enzymol.* **546**, 355-375.
Nieuwkoop, P. D. (1947). Experimental investigations on the origin and determination of the germ cells and on the development of the lateral plate and germ ridges in urodeles. *Arch. Neerl. Zool.* **8**, 1-205.
Nieuwkoop, P. D. and Faber, J. (1967). *Normal Table of Xenopus laevis (Daudin): A Systematical & Chronological Survey of the Development from the Fertilized Egg till the End of Metamorphosis*. Amsterdam: North Holland.
Nieuwkoop, P. D. and Sutasurya, L. A. (1979). *Primordial Germ Cells in the Chordates: Embryogenesis and phylogenesis*. Cambridge: Cambridge University Press.
Olson, D. J., Hulstrand, A. M. and Houston, D. W. (2012). Maternal mRNA knock-down studies: antisense experiments using the host-transfer technique in *Xenopus laevis* and *Xenopus tropicalis*. *Methods Mol. Biol.* **917**, 167-182.
Oishi, I., Yoshii, K., Miyahara, D., Kagami, H. and Tagami, T. (2016). Targeted mutagenesis in chicken using CRISPR/Cas9 system. *Sci. Rep.* **6**, 23980.
Owens, N. D. L., Blitz, I. L., Lane, M. A., Patrushev, I., Overton, J. D., Gilchrist, M. J., Cho, K. W. Y. and Khokha, M. K. (2016). Measuring absolute RNA copy numbers at high temporal resolution reveals transcriptome kinetics in development. *Cell Rep.* **14**, 632-647.
Pannese, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G. and Boncinelli, E. (1995). The *Xenopus* homologue of *Otx2* is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development* **121**, 707-720.
Papalopulu, N., Clarke, J. D., Bradley, L., Wilkinson, D., Krumlauf, R. and Holder, N. (1991). Retinoic acid causes abnormal development and segmental patterning of the anterior hindbrain in *Xenopus* embryos. *Development* **113**, 1145-1158.
Park, T. S., Lee, H. J., Kim, K. H., Kim, J.-S. and Han, J.-Y. (2014). Targeted gene knockout in chickens mediated by TALENs. *Proc. Natl. Acad. Sci. USA* **111**, 12716-12721.
Peshkin, L., Wühr, M., Pearl, E., Haas, W., Freeman, R. M., Jr, Gerhart, J. C., Klein, A. M., Horb, M., Gygi, S. P. and Kirschner, M. W. (2015). On the relationship of protein and mRNA dynamics in vertebrate embryonic development. *Dev. Cell* **35**, 383-394.
Rivera-Pérez, J. A., Mallo, M., Gendron-Maguire, M., Gridley, T. and Behringer, R. R. (1995). Goosecoid is not an essential component of the mouse gastrula organizer but is required for craniofacial and rib development. *Development* **121**, 3005-3012.
Saito, T., Pšenička, M., Goto, R., Adachi, S., Inoue, K., Arai, K. and Yamaha, E. (2014). The origin and migration of primordial germ cells in sturgeons. *PLoS ONE* **9**, e86861.
Sander, V., Reversade, B. and De Robertis, E. M. (2007). The opposing homeobox genes *Goosecoid* and *Vent1/2* self-regulate *Xenopus* patterning. *EMBO J.* **26**, 2955-2965.
Sato, T., Sakuma, T., Yokonishi, T., Katagiri, K., Kamimura, S., Ogonuki, N., Ogura, A., Yamamoto, T. and Ogawa, T. (2015). Genome editing in mouse spermatogonial stem cell lines using TALEN and double-nicking CRISPR/Cas9. *Stem Cell Rep.* **5**, 75-82.
Sekizaki, H., Takahashi, S., Tanegashima, K., Onuma, Y., Haramoto, Y. and Asashima, M. (2004). Tracing of *Xenopus tropicalis* germ plasm and presumptive primordial germ cells with the *Xenopus tropicalis* DAZ-like gene. *Dev. Dyn.* **229**, 367-372.
Shi, J., Wang, E., Milazzo, J. P., Wang, Z., Kinney, J. B. and Vakoc, C. R. (2015). Discovery of cancer drug targets by CRISPR-Cas9 screening of protein domains. *Nat. Biotechnol.* **33**, 661-667.
Sive, H. L., Grainger, R. M. and Harland, R. M. (2000). *Early Development of Xenopus laevis*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Smith, L. D. (1964). A test of the capacity of presumptive somatic cells to transform into primordial germ cells in the Mexican axolotl. *J. Exp. Zool.* **156**, 229-242.
Takahashi, G., Gurumurthy, C. B., Wada, K., Miura, H., Sato, M. and Ohtsuka, M. (2015). GONAD: Genome-editing via Oviductal Nucleic Acids Delivery system: a novel microinjection independent genome engineering method in mice. *Sci. Rep.* **5**, 11406.
van de Lavoir, M.-C., Diamond, J. H., Leighton, P. A., Mather-Love, C., Heyer, B. S., Bradshaw, R., Kerchner, A., Hooi, L. T., Gessaro, T. M., Swanberg, S. E. et al. (2006). Germline transmission of genetically modified primordial germ cells. *Nature* **441**, 766-769.
Varshney, G. K., Pei, W., LaFave, M. C., Idol, J., Xu, L., Gallardo, V., Carrington, B., Bishop, K., Jones, M., Li, M. et al. (2015). High-throughput gene targeting and phenotyping in zebrafish using CRISPR/Cas9. *Genome Res.* **25**, 1030-1042.
Véron, N., Qu, Z., Kipen, P. A. S., Hirst, C. E. and Marcelle, C. (2015). CRISPR mediated somatic cell genome engineering in the chicken. *Dev. Biol.* **407**, 68-74.
Wang, F., Shi, Z., Cui, Y., Guo, X., Shi, Y.-B. and Chen, Y. (2015). Targeted gene disruption in *Xenopus laevis* using CRISPR/Cas9. *Cell Biosci.* **5**, 15.
Wu, Y., Zhou, H., Fan, X., Zhang, Y., Zhang, M., Wang, Y., Xie, Z., Bai, M., Yin, Q., Liang, D. et al. (2015). Correction of a genetic disease by CRISPR-Cas9-mediated gene editing in mouse spermatogonial stem cells. *Cell Res.* **25**, 67-79.
Yamada, G., Mansouri, A., Torres, M., Stuart, E. T., Blum, M., Schultz, M., De Robertis, E. M. and Gruss, P. (1995). Targeted mutation of the murine goosecoid gene results in craniofacial defects and neonatal death. *Development* **121**, 2917-2922.
Yamaguchi, T., Taguchi, A., Watanabe, K. and Orii, H. (2013). DEADSouth protein localizes to germ plasm and is required for the development of primordial germ cells in *Xenopus laevis*. *Biol. Open* **2**, 191-199.
Yang, J., Aguero, T. and King, M. L. (2015). The *Xenopus* maternal-to-zygotic transition from the perspective of the germline. *Curr. Top. Dev. Biol.* **113**, 271-303.
Yoon, C., Kawakami, K. and Hopkins, N. (1997). Zebrafish *vasa* homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells. *Development* **124**, 3157-3165.
Young, J. J., Cherone, J. M., Doyon, Y., Ankoudinova, I., Faraji, F. M., Lee, A. H., Ngo, C., Guschin, D. Y., Paschon, D. E., Miller, J. C. et al. (2011). Efficient targeted gene disruption in the soma and germ line of the frog *Xenopus tropicalis* using engineered zinc-finger nucleases. *Proc. Natl. Acad. Sci. USA* **108**, 7052-7057.